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Structure–activity studies on 2-aryl-4*H*-3,1-benzoxazin-4-ones

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Eight benzoxazin-4-ones related in structure to NSC 341964 (1) have been tested for cytotoxicity in two different cell systems. Two of the benzoxazin-4-ones (3 and 10) showed good cytotoxicity (ID_{50} = 9.9 and 8.9 μM) in P388 cells. The nitrobenzoxazin-4-one (10) caused a significant alteration in cell cycle distribution when administered to P388 cells and was an inhibitor of porcine pancreatic elastase. Structure–activity relationships are discussed.

Key words: Antitumor, 2-aryl-4*H*-3,1-benzoxazin-4-ones, flow cytometry, molecular modeling, elastase inhibition.

Introduction

1-[[3-(7-chloro-4-oxo-4*H*-3,7-benzoxazin-2yl)phenyl] methyl]pyridinium chloride (**1**) (NSC 341964) has been evaluated for anti-neoplastic activity at the National Cancer Institute, Washington. The biologically active form of NSC 341964 is not known, but it is believed that the ring-opened[1] carboxylic acid (**2**) is the more likely structure. A number of structurally similar benzoxazin-4-ones have been synthesized and tested for hypocholesterolemic, hypotriglyceridemic and high-density-lipoprotein elevating properties.[2] This study[2] has also noted that facile hydrolysis of the benzoxazin-4-ones has afforded ring-opened carboxylic acids. These acids were generally as active as the parent benzoxazin-4-one in the biological test system employed. Other workers have also studied the reaction of nucleophiles (including water) with benzoxazin-4-ones.[3,4]

Benzoxazin-4-one (**1**) has been highlighted as a potential antitumor agent in some cell systems and similarly some benzoxazinones have been shown to inhibit the action of elastase enzymes.[5,6] We have therefore sought to test whether some of these hypolipidemic compounds[2] show any anti-neoplastic effects and/or elastase inhibition activities, and whether any biological activities could be related to the chemical structures. See Figure 1.

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Materials and methods

Chemicals

^{1}H- and ^{13}C-NMR spectra were determined in deuterochloroform on a Bruker AC 250 NMR Spectrometer. Chemical shifts are expressed in δ values relative to tetramethylsilane. IR spectra were recorded on a Perkin-Elmer 683 spectrometer and values are expressed in cm^{-1}. Melting points are uncorrected. Electron impact mass spectra were determined on a VG Trio-3 mass spectrometer at an ionization energy of 70 eV.

All synthetic intermediates were supplied by the Aldrich Chemical Company (Gillingham, UK). Suc-Ala-Ala-Ala-pNA and porcine pancreatic elastase were purchased from Sigma (Poole, UK). Compounds **3–8** were prepared as previously described.[2,7]

2-[4-(1,1-Dimethylethyl)phenyl]-8-methoxy-4H-benzoxazin-4-one **9.** To a solution of 2-amino-3-methoxybenzoic acid (4.17 g, 25 mM) in pyridine (38 ml) was added 4-*tert*-butylbenzoyl chloride (4.9 ml, 25 mM) dropwise over a period of 10 min. The mixture was stirred at room temperature for 3 h, poured into a mixture of ice and dilute HCl, and extracted with dichloromethane (3 × 50 ml). The combined organic phases were washed with HCl (1 M, 35 ml), water (2 × 50 ml) and dried ($MgSO_4$). Evaporation of the solvent afforded 2-[4-(1,1-dimethylethyl)benzamido-3-methoxybenzoic and (**11**) as an orange oil. ν (liquid film): 3500–3360 (N–H); 1670–1700 (C=O, acid); 1620 (C=O, amide). ^{1}H-NMR: 0.80 (9 H, s, 3 × CH_3); 4.75 (3 H, s, OMe); 6.0, 7.80 (8 H, m,

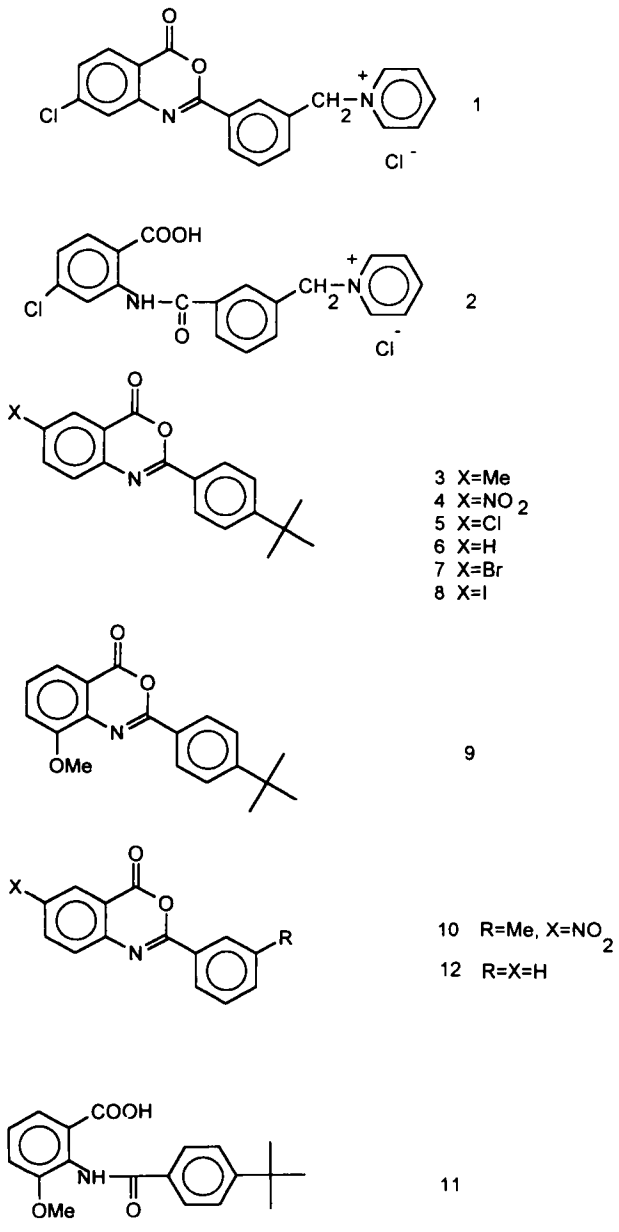

Figure 1. Structures of compounds (**1–12**).

aromatics and NH). This amide (**11**) was dissolved in acetic anhydride (63 ml) and headed under reflux for 3 h. Evaporation of the solvent afforded the title benzoxaxin-4-one (**9**) as light brown crystals (2.21 g, 29%), m.p. 105–6°C (from ethanol). *v* (KBr): 1750 (C=O); 1610 (C=N). ^{1}H-NMR: 1.20 (9 H, s, 3 × Me); 4.0 (3 H, s, OMe); 7.30 (1 H, dd, *J* = 8, 2 Hz, 7-H); 7.45 (1 H, dd, *J* = 8, 8 Hz, 6-H); 7.55, 8.25 (4 H, AA'BB' system, *J* = 8 Hz, 2', 3', 5', 6'-Hs); 7.80 (1 H, dd, *J* = 8, 2 Hz, 5-H). ^{13}C-NMR: 31.0; 35.0, 56.5, 117.2, 117.8; 119.7: 125.6; 127.4; 128.2; 137.1; 154.2; 156.2, 156.6; 159.5. M^+, 309 (70%); 162 (100%).

2-(3-Methylphenyl)-6-nitro-4H-3,1-benzoxazin-4-one **10.** To a stirred solution of 5-nitroanthranilic acid (4.52 g, 25 mM) in *N,N*-dimethylformamide (40 ml) was added dropwise triethylamine (7 ml, 50 mM) followed by 3-methylbenzoyl chloride (100 mM). After stirring for 18 h the mixture was poured into cold 2 M HCl (12 ml) and filtered to afford a yellow solid (3.86 g, 55%), m.p. 195–196°C (from ethanol). *v* (KBr): 1790 (C=O); 1610 (C=N). ^{1}H-NMR: 2.5 (3 H, s, Me); 7.45 (2 H, m, 2', 4'-Hs); 7.85 (1 H, d, *J* = 8 Hz, 8-H); 8.15 (2 H, m, 5', 6'-Hs); 8.65 (1 H, dd, *J* = 2, 8 Hz, 7-H); 9.1 (1 H, d, *J* = 2 Hz, 5-H). ^{13}C-NMR: 21.2; 117.3; 124.7; 126.1; 128.5; 128.8; 129.0; 129.3; 130.6; 134.7; 138.9; 146.3; 151.8; 157.7; 160.0. M^+, 282 (55%); 119 (100%).

Cytotoxicity testing

In vitro. Drug solutions were administered in dimethyl sulfoxide (DMSO). Cell lines tested were the murine P388 lymphocytic leukemia and the human KB epidermoid nasopharynx cell lines. The dimethyl-thiazol-diphenyltetrazolium bromide (MTT) assay[8] was used to measure drug-induced cytostasis. Values are expressed for each drug's cytotoxicity as ID_{50} (μM) (the concentration which inhibits 50% of cell growth as determined by the MTT assay). All assays were performed in sextuplicate on compounds **3–10**.

In vivo. The two most cytotoxic drugs in the P388 cell line (benzoxazin-4-ones **3** and **10**) were tested in female DBA/2 mice for *in vivo* cytotoxicity. The mice were pretreated i.p, with P388 ascitic fluid and after 4 days the drugs were administered i.p. The drugs were administered as a suspension in arachis oil at concentrations of 0, 1, 5 and 25 mg/kg in quintuplicate.

Analysis of DNA content

The effect of these benzoxazin-4-ones (**3–10**) on the DNA content of P388 cells was measured by flow cytometry as described previously.[9] Briefly, the cells were treated with drug (10 μg/ml), followed by fixation in cold acetone:ethanol (50:50, 4°C) after 1, 2, 3, 4, 5, 6, 7, 12 and 26 h. The cells were rehydrated and washed in phosphate buffered saline (pH 7.0), before staining with propidium iodide (50 μg/ml). DNA content was measured by flow cytometry (Coulter Epics V) using an argon

laser (λ_{ex} = 488 nm, $\lambda_{em} \geq$ 500 nm). Coefficients of variation were less than 6% and the fraction of cells in each phase of the cell cycle was calculated using the commercial software provided by Coulter. Over the course of 26 h the percentage of cells in each phase of the cell cycle in untreated cells does not vary.

Molecular modeling

The benzoxazinones (**3–10** and **12**) were built using the Quanta program (Molecular Simulations, Sunnyvale, CA). The structures were minimized using the Steepest Descents algorithm followed by the Newton–Raphson method provided by the Charmm program (Molecular Simulations). Conformational studies on the minimized benzoxazin-4-ones (**3–10** and **12**) were carried out using the Conformational Search program from Quanta. The torsion angle of the benzoxazin-4-one-aryl bond was altered stepwise by 10° and minimized in these fixed positions by the Adopted Basis Newton–Raphson procedure following each step. This process afforded graphs of torsion angle versus potential energy for each compound (**3–10** and **12**) which were analyzed to discover the conformations of the benzoxazinones (**3–10** and **12**) at different potential energies.

Atomic charge distribution over the benzoxazinones (**3–10** and **12**) was measured by using the CINDO program provided with the Quanta software.

To study the possibility that a benzoxazinone may fit into the porcine pancreatic elastase (PPE) binding site, a crystal structure of an isocoumarin in its PPE binding site[10] was obtained from the Brookhaven Protein Databank via the Chemical DataBank Service (Daresbury, Warrington, UK). Using the Molecular Similarity Module from Quanta the oxazine ring of the benzoxazinone (**10**) was overlaid with the equivalent atoms from the isocoumarin in its active site. After overlapping, the isocoumarin was removed using Quanta's Molecular Editor and the resulting benzoxazinone–PPE complex was minimized using the Steepest Descents program (30 iterations). The initial isocoumarin–PPE complex is in a high energy state and to make a comparison with the modeled benzoxazinone–PPE complex this crystal structure was minimized (Steepest Descents, 30 iterations). The isocoumarin and benzoxazinone in the PPE active site were then examined using the geometric programs available in Quanta.

Elastase inhibition studies

The ability of the benzoxazinones (**3–10**) to inhibit the action of pancreatic elastase was determined by the method previously described.[6] Briefly the enzyme (0.05 mg/ml) was dissolved in 1 mM HCl; the substrate (Suc-Ala-Ala-Ala-pNA) was dissolved in 0.05 mM Tris–HCl (pH 8.0) and the benzoxazinones were prepared in DMSO.

Hydrolysis of the substrate was measured by recording the release of *p*-nitroaniline at 405 nm at 25°C. For inhibition of elastase a UV cell containing 1.68 ml buffer solution and 0.2 ml substrate (0.68 mM) and 0.02 ml inhibitor solution (0–250 μM) was incubated at 25°C. Hydrolysis was started by the addition of 0.1 ml enzyme solution and the increase in absorbance (*I*) at 405 nm was measured over 0–30 min. From these experiments a graph of ΔI/min versus inhibitor concentration was obtained. From this graph the amount of inhibitor required to halve the rate of hydrolysis (ID_{50}) could be measured for the benzoxazinones which showed inhibitory activity.

Results

The *in vitro* cytotoxicities of benzoxazin-4-ones **3–10** in P388 and KB cells are depicted in Table 1. Compounds **3** and **10** were the most cytotoxic (ID_{50} = 9.9 and 8.9 μM, respectively) in the P388 cell line, and were at least twice as cytotoxic as the other compounds (**4–9**) in this series. The benzoxazin-4-ones were generally less cytotoxic in the KB cell line. Also, the individual cytotoxicities of the compounds **3–10** were less in the KB cell line in comparison with the P388 cell line.

The only drug in this series (**3–10**) which caused a significant alteration in cell cycle distribution was

Table 1. Cytotoxicities of benzoxazinones (**3–10**) in P388 and KB cells

Compound	P388	KB
3	9.9	47.8
4	25.0	25.3
5	21.7	70.5
6	25.4	24.4
7	19.0	31.6
8	18.5	25.9
9	>97	42.7
10	8.9	26.2

The figures refer to the ID_{50} (μM) for each compound.

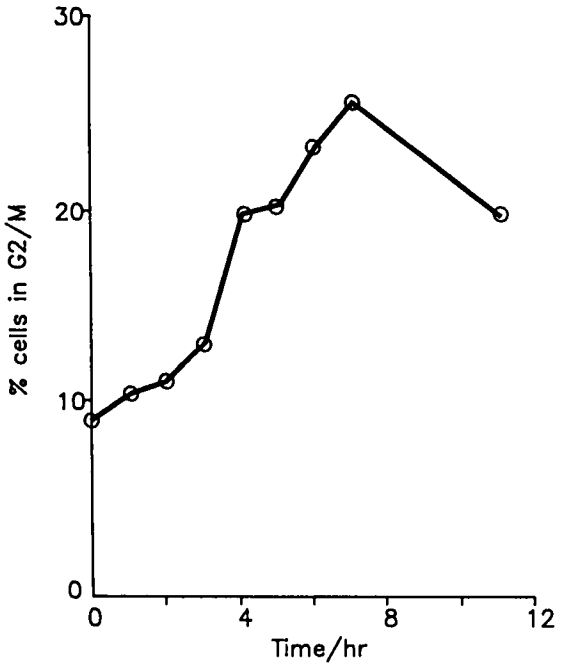

Figure 2. Accumulation of P388 cells in the G_2/M phase of the cell cycle following treatment with benzoxazin-4-one (**10**) (10 μg/ml).

the nitrobenzoaxazin-4-one (**10**). This agent (**10**) caused an increase in the proportion of cells in the G_2/M phase of the cell cycle (Figure 2). An increase (2-fold) was still apparent 26 h after treatment (data not shown).

The treatment of female DBA/2 mice which had previously been treated with ascitic P388 cells i.p. with compounds **3** and **10** up to a dose of 25 mg/kg produced no significant increase in life span (ILS) over the control mice.

Molecular modelling studies show that the lowest energy conformations of all the benzoxazinones (**3–10** and **12**) are those in which the benzoxazinone skeleton and the 2-aryl groups are co-planar. Deviation from co-planarity increases the energy as these two groups become more perpendicular to each other. The CINDO calculations show that the carbonyl carbons possess an atomic charge of +0.41 for each benzoxazinone (**3–10** and **12**).

The modelling of the benzoxazinone (**10**) in the PPE active site shows that the 195 serine hydroxyl (the hydrolyzing moiety) is 2.92 Å from its target (the carbonyl carbon of the benzoxazinone). The distance between the 195 serine hydroxyl and the hydrolyzed carbonyl carbon of the ring opened isocoumarin is also 2.92 Å.

Only compounds **6**, **7** and **10** showed significant elastase inhibition properties. The ID_{50}s were 26, 40 and 63 μM, respectively.

Discussion

All the benzoxazin-4-ones tested here except for compound **10** have a *tert*-butyl group attached to the phenyl ring. Of the eight benzoxazin-4-ones (**3–10**) tested, the *tert*-butyl derivative (**3**) and compound (**10**) showed the most potent cytotoxicity in P388 lymphocytic leukemia cells (ID_{50} = 9.9 and 8.9 μM). The halogenated derivatives (**5**, **7** and **8**), the unsubstituted benzoxazin-4-one (**6**) and the nitro compound (**4**) show similar cytotoxicities (ID_{50} = 18.5–25 μM) in P388 cells. The methoxy-substituted benzoxazin-4-one (**9**) was essentially non-toxic in P388 cells.

In the KB cell line benzoxazin-4-one (**10**) was the second most cytotoxic compound in this series (ID_{50}=26.2 μM), whilst the methyl derivative (**3**) was relatively non-toxic (ID_{50}=47.8 μM).

The mechanism by which these agents (**3–10**) exert their cytotoxic effects is unknown, although they have been shown to possess atherogenic activity.[2]

Cell cycle analysis of P388 cells following treatment with benzoxazin-4-ones (**3–10**) showed that only one agent (**10**) caused a significant perturbation in cell kinetics. This was an increase in cells in the G_2/M phase of the cell cycle. A block in this phase of the cell cycle can arise as a result of damage to a variety of cellular targets such as DNA or proteins (e.g. tubulin). These agents, although similar in structure to antifolates such as aminopterin and methotrexate, are, however, unlikely to be acting as antimetabolites as the characteristic increase in the percentage of cells in the S phase of the cell cycle was not observed.

Molecular modelling studies show that rotation about the benzoxazin-4-one–aryl bond in compounds (**3–10** and **12**) can occur but the difference between the minimum and maximum conformational energies is high (4–10 kcal/mol). This means that at 25°C the probability (Boltzmann) of finding the molecule in the highest energy conformation is virtually zero. In all cases the lowest energy is found where the benzoxazin-4-one and aryl rings are co-planar. This low energy conformation is a consequence of the fact that there are no groups on the 1 and 3 positions of the benzoxazin-4-one which could clash with the two 2′- and 6′-aryl protons. Also, with the two ring systems co-planar electronic conjugation is conserved. A substituent on the fused

benzene ring is unlikely to have any significant effect on the conformation of the benzoxazin-4-one–aryl bond. This study indicates that substituents on the 3′ and 4′ positions of the rotatable aryl ring do not distort the co-planarity of the tricyclic system.

Serine proteases such as elastase and α-chymotrypsin have been shown to cause metastatic migration of rat ascites tumor cells to the lung.[11] Elastase enzymes catalyze the hydrolysis of amide bonds by transferring the acyl part of an amide (or ester) to a serine hydroxyl in the active site of the enzyme. (Mechanistically this is similar to basic hydrolysis as opposed to acidic hydrolysis of an amide.) The CINDO calculations indicate that the site of hydrolysis (the carbonyl atom) possesses an atomic charge of +0.41 for all the compounds (**3–10** and **12**). This suggests that the atomic charge on the acyl group of the benzoxazinones (**3–10** and **12**) is not the reason for their differential elastase inhibition activities.

Modelling of benzoxazinone (**10**) in the active site (Figure 3) of the PPE enzyme[10] has shown that the active serine hydroxyl is 2.92 Å distant from its hydrolysis target (the carbonyl carbon of the benzoxazinone). This is the same distance found in the equivalent atoms of the isocoumarin–PPE complex. This suggests that benzoxazinone (**10**) can share the same binding site as a structurally similar isocoumarin and that the benzoxazinone skeleton is a good candidate for inhibiting the action of the elastase enzyme. This compound (**10**) is an inhibitor of elastase.

With the *tert*-butyl substituted compounds (**3–9**), the benzoxazin-4-one (**3**) with an electron donating substituent (CH_3 group) on the six position was the most cytotoxic in P388 cells. Benzoxazin-4-ones with neutral electronic effects (**6**) and with electron withdrawing substituents (**4**, **5**, **7** and **8**) in the six position were less cytotoxic. However, benzoxazin-4-one (**9**) with an electron donating methoxy substituent in the eight position was the least cytotoxic. The benzoxazin-4-one (**10**) with a methyl group attached to the aryl ring and a electron withdrawing (nitro) group in the six position showed the greatest cytotoxicity in P388 cells.

The unsubstituted benzoxazinone (**6**) showed the greatest elastase inhibition activity (ID_{50} = 26 μM), followed by the brominated compound (**7**). However, the cytotoxic nitro substituted benzoxazinone (**10**) also showed good elastase inhibitory activity (ID_{50} = 63 μM). This compound (**10**) also caused an increase in cells in the G_2/M phase of the cell cycle.

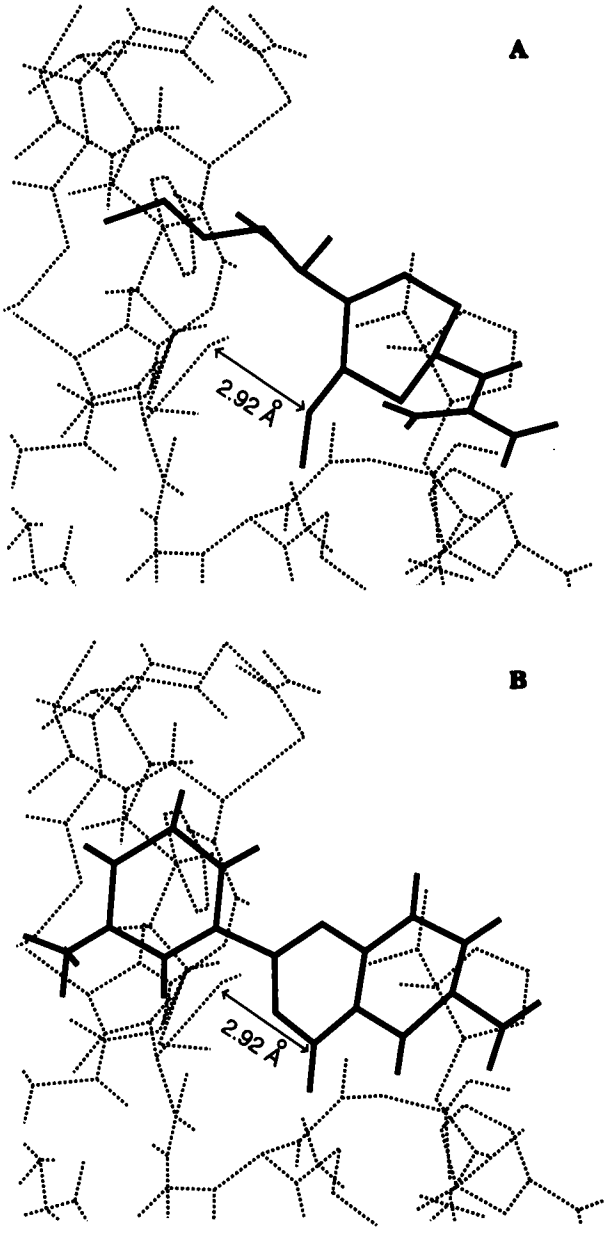

Figure 3. (a) Structure of the PPE (dotted lines)–isocoumarin (bold lines) complex. (b) Structure of the PPE (dotted lines)–benzoxazinone (**10**) (bold lines) complex. Both structures show the separation (2.92 Å) between the serine hydroxyl and the acyl carbon ligand.

Conclusions

In the cases considered here a range of cytotoxicities was noted (see Table 1). For effective cytotoxicity it appears that the *tert*-butyl substituted benzoxazin-4-ones require an electron donating substituent in the six position. The conclusion that can be drawn about the benzoxazin-4-one (**10**)

without a *tert*-butyl substituent on the aryl ring is that an electron withdrawing group on the six position provides a relatively cytotoxic compound. This compound (**10**) amongst the other benzoxazinones tested here has uniquely shown the ability to cause a significant perturbation in cell cycle kinetics as well to be an effective inhibitor of elastase. These properties together suggest that the nitrobenzoxazinone (**10**) may be a useful lead compound as an anticancer drug.

Molecular modelling studies have indicated that the tricyclic 2-aryl-3,1-benzoxazin-4-one system is co-planar and that the benzoxazinone skeleton fits the active site of the PPE enzyme.

Changes in the substituents and to their regiochemistry are more likely to lead to a more biologically active benzoxazin-4-one than by trying to force the benzoxazin-4-one–aryl bond to adopt a different conformation (by, for example, inserting a bulky group on the 2′ and/or 6′ positions). With these general conclusions in mind, studies are underway to further refine the parameters associated with cytotoxicity, elastase inhibitory action and cell cycle perturbation activity of 2-aryl-benzoxazin-4-ones.

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