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The Molecular Components of Nitrofurantoin Critical to Its Inhibitory Effect upon Platelet Aggregation

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SUMMARY

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Nine analogues of nitrofurantoin were studied in order to determine the molecular components critical to its inhibitory effect upon primary ADP-induced platelet aggregation. The structural alterations represented by these analogues indicate that the inhibitory effect of nitrofurantoin is due to the simultaneous presence of the nitro group on the furan ring and the specific arrangement of the diketo groups on the imidazole ring. Molecular orbital calculations predicted the key role of the electron-deficient nitrofurantoin moiety in the inhibitory effect. A specific ADP-nitrofurantoin complex was demonstrated in solution by nuclear magnetic resonance spectroscopy, but the dissociation constant for the complex (approximately 10^{-1} M) cannot account for the competitive inhibitory effect of nitrofurantoin on platelet aggregation ($K_i \approx 10^{-5}$ M). The over-all data suggest that the nitrofuran and diketoimidazole portions of nitrofurantoin contribute to its inhibitory effect through different mechanisms. These studies also suggest a set of molecular requirements for the synthesis of new inhibitors which could be clinically useful.

INTRODUCTION

There is considerable evidence suggesting that platelet aggregate formation may be the immediate cause of the thromboses and thromboembolic phenomena associated with arterial vascular disease (1-3). Occluding intravascular platelet aggregated conceivably might be prevented by drugs which inhibit platelet adherence to surfaces, the platelet release reaction, or platelet aggregation induced by ADP (4). Sulfinpyrazone (5) and aspirin (6) are clinically useful inhibitors of the platelet release reaction. However, an inhibitor of ADP-induced platelet aggregation is not yet available for use in human subjects. Recently we reported that nitrofurantoin is an inhibitor of primary ADP-induced platelet aggregation at concentrations that can be achieved *in vivo* following oral administration (7). This report identifies the molecular components of nitrofurantoin critical to its inhibitory effect and describes data relating to its pharmacological mechanism of action.

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METHODS

Blood was drawn from human volunteers and placed in 3.8 g/100 ml of trisodium citrate in a ratio of 9:1. Platelet-rich plasma was prepared as described previously (7).

Materials. Nitrofurantoin [1-(5-nitro-2'-furfurylideneamino)-2,4-deketoimidazole] and nine analogues (Fig. 1) were provided by the Norwich Pharmacal Company. They were added to water, and the actual concentrations achieved were determined spectrophotometrically (except for analogue IX, which was completely soluble) utilizing the appropriate extinction coefficient as provided by Norwich. These stock solutions were diluted to their final concentrations in 100 mM Tris-HCl (pH 7.5), 95 mM NaCl, and 3 mM KCl. Solutions of ADP and collagen were prepared as described previously (7, 8).

Deuterium oxide (99.97%) NaOD, and sodium 2,2-dimethyl-2-silapentanesulfonate were purchased from Wilmad Glass Company, Buena, N.J.

Platelet aggregation studies. Platelet aggregation studies were performed according to Born (9). Both 0.25 ml of platelet-rich plasma and 0.02 ml of analogue solution or buffer (control) were placed in a 1.5-ml siliconized cuvette containing a metal stir bar. The cuvette was then inserted into a platelet aggregometer (Chrono-Log Corporation, Broomall, Pa.) set at 37° with a stirring speed of 1100 rpm. The light transmission through the samples of platelet-rich plasma were inscribed on an EU-20B servo recorder (Health/Schlumberger Scientific Instruments Division, Heath Company, Benton Harbor, Mich.). ADP or collagen was added to the plasma as it was being stirred. This produced an increase in light transmission which was proportional to the amount of platelet aggregation. The aggregation responses obtained with ADP (1, 1.5, and 2 μM) or collagen in the presence of an analogue (24–150 μM) were compared with those obtained with buffer alone or with 24 μM nitrofurantoin. We employed a collagen concentration that was near the threshold required to induce platelet aggregation. Because of the marked individual variation in platelet responsiveness to collagen (8), the actual concentration of collagen employed ranged between 0.2 and 0.8 μg/ml. Each analogue was tested on samples of platelet-rich plasma obtained from at least three different individuals.

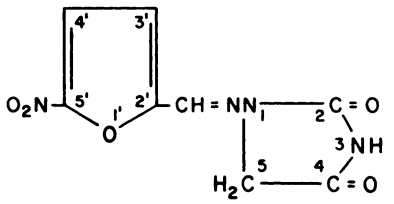


FIG. 1. *1-(5'-Nitro-2'-furfurylideneamino)-2,4-diketoimidazole (nitrofurantoin, parent compound)*

The nine analogues of nitrofurantoin studied were: I, 1-(5'-nitro-2'-furfurylideneamino)-2-ketoimidazole; II, 1-(5'-nitro-2'-furfurylideneamino)-2,5-diketoimidazole; III, 1-(5'-nitro-2'-furfurylideneamino)-2-imino-4-ketoimidazole; IV, 1-(5'-nitro-2'-furfurylideneamino)-2-thioketo-4-ketoimidazole; V, 1-(5'-nitro-2'-furfurylideneamino)-3-methyl-2,4-diketoimidazole; VI, 1-(2'-furfurylideneamino)-2,4-diketoimidazole; VII, 1-(5'-amino-2'-furfurylideneamino)-2,4-diketoimidazole; VIII, 5-nitro-2-furoic acid; IX, 1-amino-2,4-diketoimidazole.

Molecular orbital calculations. Quantum mechanical calculations were made by the semiempirical Hükel method (10). Only the π-electrons of the unsaturated portion of the molecule are considered. In this type of analysis the σ-bond framework of these closely related analogues is assumed to be constant and the π-electrons are treated separately. The values for the empirical parameters α and β, substituted for the coulomb and resonance integrals, respectively, were those proposed by the Pullmans (11). In the analogues containing a nitro group, nitrogen has a formal charge of +1. Therefore an α_N of 2.0 and β_{N-O} of 0.7 were used as suggested by Streitwieser (12). One p-π-orbital from each oxygen and the nitrogen were considered, with a total of 4 electrons.

Calculations of electronic charge distribution and the energies of the highest occupied and lowest empty molecular orbitals were made with a simple Hückel program, implemented on a model 6400 Control Data Corporation computer. HOMO and

LEMO[1] values are reported in relative β-units.

Nuclear magnetic resonance studies. Nuclear magnetic resonance spectra were recorded on a Hitachi Perkin-Elmer (R20-B 60 MHz) spectrometer operated at an ambient temperature of 35°. The "pH" of solutions in D_2O was adjusted with NaOD, and the values are reported as pD (pD = pH_{obs} + 0.4) (13).

RESULTS

Platelet aggregation studies. Analogues I–VII represent specific alterations of the nitrofurantoin structure. Aggregation tracings obtained in the presence of 24 μM concentrations of analogues I–VII were compared with those obtained in the presence of 24 μM nitrofurantoin (N) or buffer alone (C) (see Fig. 2). Each study confirmed the inhibitory effect of nitrofurantoin and demonstrated the critical nature of a specific molecular alteration. When analogues I–VII were present in concentrations which approached their solubility limits (100–150 μM), the aggregation curves still did not differ from controls.

Various alterations of the 2, 4-diketoimidazole portion of nitrofurantoin resulted in derivatives which had virtually no effect upon platelet aggregation. These alterations included deletion of 4-keto moiety (analogue I), shift of the keto moiety in position 4 to position 5 (analogue II), and substitution of the 2-keto moiety by an imino (analogue III) or thioketo (analogue IV) moiety. Substitution of a methyl group on position 3 of the imidazole ring (analogue V) also diminished the inhibitory effect. The nitro group on the furan ring was also critical to the inhibitory effect of nitrofurantoin upon platelet aggregation. Its deletion (analogue VI) or substitution by an amino group (analogue VII) completely eliminated any inhibitory effect. Figure 2 depicts representative tracings obtained with analogues I and VI.

In order to assess the individual contributions of the nitrofuran and diketoimidazole moieties to the inhibitory effect of nitrofurantoin, we studied the effect upon platelet aggregation of analogues VIII and IX (5-nitro-2-furoic acid and 1-amino-2,4-diketoimidazole, respectively), which represent the two halves of the nitrofurantoin molecule. Both analogues were ineffective at 24 μM. However, in contrast to analogues I–VII, analogues VIII and IX were soluble and could be tested at much higher concentrations. In final concentrations of 2.5 mM, 5-nitro-2-furoic acid was effective whereas 1-amino-2,4-diketoimidazole was still virtually ineffective (four experiments) (Fig. 3). These studies suggest that the nitrofuran moiety is of major importance to the inhibitory effect of nitrofurantoin upon platelet aggregation.

Molecular orbital calculations. In order to examine the over-all electronic nature of the molecule and assess the individual contributions of the critical parts, we performed molecular orbital calculations on nitrofurantoin and several of its analogues (14). Table 1 lists the various analogues and the calculated energy values for the LEMO, which is a measure of electron-accepting ability, and the HOMO, which is a measure of electron-donating ability. Ta-

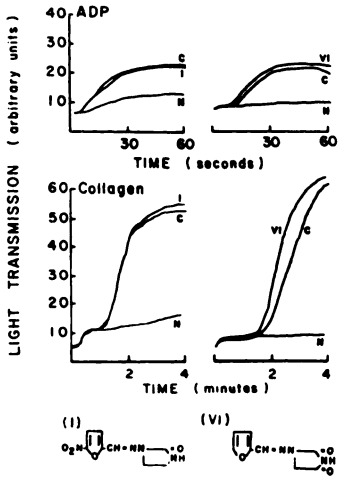


FIG. 2. *Effects of nitrofurantoin (N) and analogues I and VI upon ADP (1 μM)- and collagen-induced platelet aggregation*

C = control tracing containing buffer only. The final concentration of nitrofurantoin and analogues I and VI in these tracings was 24 μM.

[1] The abbreviations used are: HOMO, highest occupied molecular orbital; LEMO, lowest empty molecular orbital.

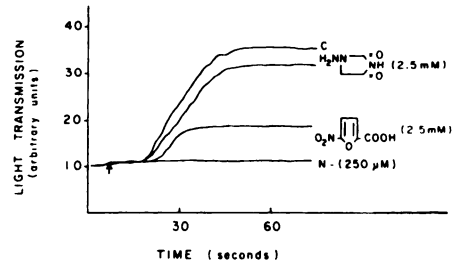


FIG. 3. *Effects of 250 μM nitrofurantoin (N), 2.5 mM 5-nitro-2-furoic acid (analogue VIII), and 2.5 mM 1-amino-2,4-diketoimidazole (analogue IX) upon ADP-induced platelet aggregation*

C = control tracing containing buffer only. The arrow indicates the point of injection of ADP (final concentration, 1.5 μM). Note that at 2.5 mM 5-nitro-2-furoic acid is more potent than 1-amino-2,4-diketoimidazole as an inhibitor of platelet aggregation.

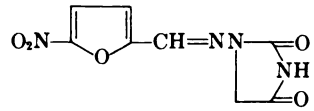
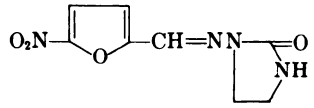
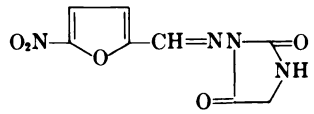
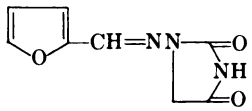
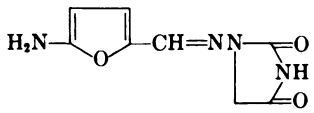
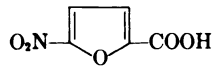
ble 1 also shows the partial "charge" (i.e., π-electron deficiency or surplus) on the atoms of the furan ring. Nitrofurantoin has a low-energy LEMO, indicating potential as an electron acceptor, and its furan ring has a net partial positive charge. Alterations in the diketoimidazole portion of the molecule (analogues I and II) did not significantly change the LEMO and HOMO energies or the net partial charge on the furan ring. However, deletion of the nitro group (analogue VI) or its substitution by an amino group (analogue VII) increased the LEMO and HOMO energies and conferred a net partial negative charge on the furan ring. 5-Nitro-2-furoic acid had the lowest LEMO energy and the largest net partial positive charge on the furan ring, while 1-amino-2,4-diketoimidazole had a very high-energy LEMO and a high-energy HOMO, indicating its potential as an electron donor.

Nuclear magnetic resonance studies. We reported previously (7) that nitrofurantoin is a competitive inhibitor of ADP-induced platelet aggregation, with an inhibitor dissociation constant $K_i \approx 10^{-5}$ M. However, the mechanism of the competitive effect was not established. Since nitrofurantoin is not an obvious structural analogue of ADP, the classical interpretation of competitive inhibition (namely, competition between antagonist and agonist for the same binding site) could not be invoked easily. Moreover, the alternative interpretation (namely, that the antagonist may combine directly with the agonist) stood as a plausible model in this case owing to the nature of the ADP and nitrofurantoin molecules. Molecular orbital calculations indicate that ADP and nitrofurantoin may form a charge-transfer type complex. ADP may act as an electron donor [adenine has a relatively high-energy HOMO (15)] while nitrofurantoin may act as an electron acceptor [nitrofurantoin has a relatively low-energy LEMO (Table 1)]. Therefore it was necessary to determine whether ADP and nitrofurantoin form a complex and, if so, whether the complex is strong enough to account for the inhibitory effect of nitrofurantoin.

Nuclear magnetic resonance spectroscopy is a valuable technique for characterizing a complex involving aromatic molecules, because the spectral changes observed are specific and characteristic of the nature and strength of the complex (16, 17). Figure 4 shows that a specific complex is formed between ADP and nitrofurantoin. The positions of the signals corresponding to protons attached to the nitrofuran moiety of nitrofurantoin are markedly affected (see arrows) when ADP is added to the solution. The magitude of these changes in signal position ($\Delta\delta$) is dependent upon ADP concentration (Table 2). All the nitrofurantoin signals were displaced upfield. This is consistent with the presence of a complex in which the adenine ring of ADP is parallel to the nitrofuran ring of nitrofurantoin (a ring-stacking orientation). Such an orientation would allow the most favorable overlap of the π-orbitals of the two molecules. The amount of change in signal position ($\Delta\delta$) is different for the different protons *b, c, d*. These results indicate that the mutual orientation of the two molecules in complex is such that protons *b, c,* and *d* lie at different distances from the center of the adenine ring. Figure 5 presents a double-reciprocal plot of the data shown in Table 2. The common intersection on the abscissa confirms that the changes in each of the signals of nitrofurantoin reflect the same interaction with ADP. Moreover, this point

TABLE 1

Electronic properties of nitrofurantoin and its analogues

Compound	LEMO	HOMO	"Charge" on furan atoms[a]					
			O_1	C_2	C_3	C_4	C_5	Net "charge"
	rel. β-units							
 Nitrofurantoin	−0.1190	−0.6038	0.200	0.184	0.082	0.017	0.296	0.779
 Analogue I	−0.1181	−0.6034	0.208	0.183	0.082	0.017	0.296	0.786
 Analogue II	−0.1436	−0.6326	0.002	0.194	0.141	0.076	0.315	0.728
 Analogue VI	0.6962	−0.2625	0.202	−0.014	−0.119	−0.098	−0.022	−0.051
 Analogue VII	0.7647	−0.1081	0.229	−0.083	−0.099	−0.208	−0.077	−0.084
 5-Nitro-2-furoic acid	−0.3197	−0.8414	0.238	0.302	0.009	0.147	0.255	0.951
 1-Amino-2,4-diketoimidazole	1.5757	−0.0873						

[a] Values represent the relative π-electron densities at the various atoms and the net partial "charge" on the furan ring.

of intersection provides an estimate of the dissociation constant for the complex (K_{diss} = 1.6 × 10^{-1} M).

5-Nitrofuroic acid (analogue VIII) was also tested for direct interaction with ADP. At pD 9.2 there was an ADP-dependent upfield displacement of the two nitrofuroic acid signals, indicating complex formation with K_{diss} = 6.3 × 10^{-1} M (Fig. 6). Thus, under the same conditions, the nitro-

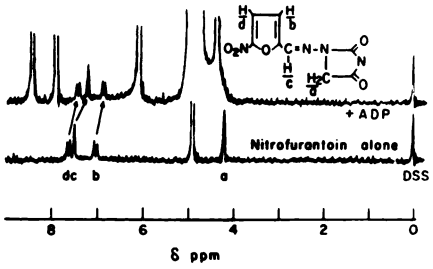


FIG. 4. *NMR spectra of nitrofurantoin in the presence and absence of ADP*

The lower spectrum is that of 0.04 M sodium nitrofurantoin in D_2O, pD ~ 9.2. The lower-case letters show the assignment of specific signals in the spectrum to specific protons on the nitrofurantoin molecule. The large signal at δ = 4.8 ppm corresponds to residual HDO in the D_2O solution. The upper spectrum is that of the cosolution of 0.04 M nitrofurantoin and 0.60 M ADP in D_2O, pD ~ 9.2. The two spectra are aligned with respect to the sodium 2,2-dimethyl-2-silapentanesulfonate (DSS) internal reference of 0 ppm. The additional signals, cut off in the upper spectrum, correspond to protons on ADP, which was present in excess. The arrows depict the changes in position of the nitrofurantoin signals indicative of complex formation with ADP.

[A signal for the methylene protons *a* of nitrofurantoin is not apparent in the upper spectrum for two reasons: (a) it is obscured by the presence of the large signal due to ADP, and (b) it is greatly diminished in magnitude because of exchange. The presence of the high concentration of buffer (i.e., the PP_i moiety of ADP) accelerates the rate of exchange of these relatively acidic protons with deuterium from the solvent, D_2O. Since deuterium does not absorb in this frequency range, the signal is diminished as the protium atoms are replaced by deuterium. The half-time for this process, determined in a separate experiment, was approximately 20 min. For these reasons signal *a* was not included in the protocols for determination of the K_{diss} for the complex (Table 2 and Fig. 5).]

furoic acid–ADP interaction was approximately 4 times weaker than the nitrofurantoin-ADP interaction. At pD 7.8 the nitrofuroic acid–ADP interaction was increased to $K_{diss} = 4.5 \times 10^{-1}$ M (data not shown. When 1-amino-2,4-diketoimidazole (analogue IX) was tested at pD 7.8, there was no significant change in the position of the NMR signal as a function of ADP concentration (data not shown). This indicates that the "other half" of the nitrofurantoin molecule (analogue IX) does not interact with ADP.

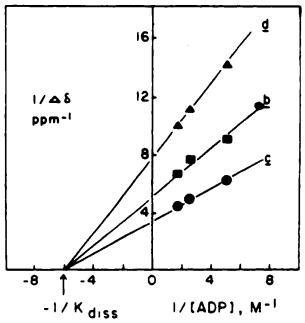


FIG. 5. *Double-reciprocal plot of dependence of nitrofurantoin NMR signal positions on ADP concentration*

The data are those of Table 2. The letters *b*, *c*, and *d* refer to the specific nitrofurantoin signals. The intercept on the abscissa corresponds to $-1/K_{diss}$.

TABLE 2

Variation of nitrofurantoin NMR signal positions with ADP concentration

All solutions were in D_2O, pD≈9.2. At lower pD values nitrofurantoin was relatively insoluble. Individual stock solutions of ADP, nitrofurantoin, and K_2HPO_4 (P_i) were prepared in D_2O, adjusted to pD~9.2 if necessary, and then combined in the proper ratios to give the relative concentrations shown. The phosphate was added to maintain the ionic strength relatively constant as the ADP concentration was varied. The pD values of the individual solutions were rechecked after the NMR spectra were recorded, and they were all found to be within the range pD 9.1–9.3. All spectra were recorded at the ambient temperature of the instrument, approximately 35°. Signal positions (δ) were measured with respect to the internal reference, sodium 2,2-dimethyl-2-silapentanesulfonate, which was set at 0 ppm in all cases, and individual signal positions are considered accurate to ±0.01 ppm. The $\Delta\delta$ values represent the difference between the position of a particular signal in sample 0 (control) and the particular sample in question, e.g., $\Delta\delta_b^1 = (\delta_b^0 - \delta_b^1)$.

Sample	ADP	Nitrofurantoin	P_i	Change in position ($\Delta\delta$) of nitrofurantoin signals		
				b[a]	*c*	*d*
	M	*M*	*M*	*ppm*		
0	0	0.04	1.2			
1	0.033	0.04	1.13	0.03	0.05	0.01
2	0.20	0.04	0.8	0.11	0.16	0.07
3	0.40	0.04	0.4	0.13	0.20	0.09
4	0.60	0.04	0	0.15	0.22	0.10

[a] Letters correspond to the signals as they are labeled in Fig. 4.

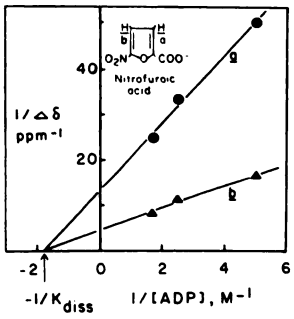


FIG. 6. *Double-reciprocal plot of dependence of nitrofuroic acid NMR signal positions on ADP concentration*

The experiment was conducted under the conditions described for Table 2, except that nitrofuroic acid was substituted for nitrofurantoin. Only two signals (*a* and *b*) occur in the NMR spectrum of nitrofuroic acid, and changes in their positions with respect to ADP concentration were measured as described in Table 2 and used to plot this graph.

DISCUSSION

The studies of nitrofurantoin analogues indicate that the inhibitory effect of nitrofurantoin upon platelet aggregation is due to the simultaneous presence of the nitro group on the furan ring and the specific arrangements of the diketo groups on the imidazole ring. The nitro group is an electron acceptor which withdraws electrons from the furan ring. Thus the nitrofuran portion of the molecule is highly polar in nature, consisting of an electronegative nitro group associated with an electropositive furan ring. Deletion of the nitro group removes this polarity, while substitution of the nitro group by an electron donor such as an amino group reverses the polarity (i.e., electropositive amino group and electronegative furan ring). These changes (analogues VI and VII) result in molecules which have a high LEMO energy and lack inhibitory action on platelet aggregtion. These demonstrate the importance of the nitro group and strongly suggest that its contribution to the inhibitory effect is mediated through the electronic characteristics it confers upon the nitrofurantoin molecule.

The greater effectiveness of 5-nitro-2-furoic acid relative to 1-amino-2,4-diketoimidazole is probably attributable to the nitro group, although activity associated with the new functional group (i.e., carboxyl residue) cannot be ruled out. However, despite the low LEMO energy of 5-nitro-2-furoic acid, it is markedly less potent than nitrofurantoin (Fig. 3). Moreover, analogues which retain the nitro group and have low LEMO energies can be rendered ineffective by alterations in the diketoimidazole portion of the molecule. These findings indicate that the electronic effects of the nitro group are not totally responsible for the inhibitory effect of nitrofurantoin, and that the diketoimidazole group facilitates inhibition through some mechanism other than its contribution to the over-all electronic character of the molecule.

The NMR studies on nitrofurantoin were conducted at pD 9.2 and at relatively high ionic strength because of difficulties in dissolution under less alkaline conditions. A shift to neutral conditions and to lower ionic strength would be expected to enhance the ADP-nitrofurantoin interaction. Such an enhancement was noted in our studies when the ADP–nitrofuroic acid complexes were studied at pD 7.8 rather than pD 9.2. However, the degree of enhancement associated with decline in pD was slight. It could not be expected to change the K_{diss} of the ADP-nitrofurantoin complex (1.6×10^{-1} M) by the approximately 4 orders of magnitude that would be required before this interaction could be invoked to explain the competitive inhibitory effect of nitrofurantoin upon ADP-induced platelet aggregation [$K_i \approx 10^{-5}$ M (7)]. Therefore we must conclude that nitrofurantoin does not act primarily by complexing the free ADP in plasma and thereby depleting the number of ADP molecules available to bind with platelets. However, nitrofurantoin may bind at a site on the platelet surface close enough to the bound ADP so that the two molecules interact and the aggregating action of ADP becomes altered. In this regard it is interesting that the order of increasing strength of interaction with ADP (as determined by the NMR studies) is 1-amino-2,4-diketoimidazole < 5-nitrofuroic acid < nitrofurantoin. This order parallels the order of inhibitory potency for these compounds.

The exact contribution of the nitrofuran

and diketoimidazole moieties to the inhibitory effect of nitrofurantoin must await further studies. However, the identification of the molecular components of nitrofurantoin critical to its inhibitory effect does establish a set of molecular requirements for the synthesis of new inhibitors of this type: an electron-deficient site coupled to a structure carrying two keto groups in a specific spatial conformation. New drugs substituting a different electron-withdrawing group for the nitro group would be of particular interest, since nitroaromatic derivatives are implicated in various types of toxic reactions (18). Such drugs would lack the toxic risks associated with the nitrofuran moiety but would retain the unique electronic nature. This type of approach may lead to the discovery of a clinically useful inhibitor of ADP-induced platelet aggregation.

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