BMY21190, A Potent Inhibitor of cAMP Phosphodiesterase

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INTRODUCTION

A number of compounds capable of inhibiting the aggregation of blood platelets have
been reported in recent years. Synthesized compounds containing the imidazo [4,5-b]
quinoline-2-one nucleus are a new series of structurally-novel inhibitors of platelet ag-
gregation (38). These compounds are potent broad-spectrum inhibitors that inhibit platelet
aggregation caused by thrombin, ADP, and collagen with roughly equal potency (8,15).
BMY21190 (Fig. 1) inhibits ADP- and collagen-induced aggregation of rabbit platelets
with an efficacy comparable to prostacyclin (38). BMY21190 may also be capable of
elevating the cAMP levels within platelets sufficiently to activate the cAMP-dependent
protein kinase as does BMY20844 (60, Fig. 1). The active catalytic subunit is released by
the binding of cAMP to the regulatory subunit of the protein kinase and then acts at many
different steps in the biochemical pathways to inhibit platelet inhibition (13,58,59).
Agents that elevate platelet cAMP levels, including inhibitors of platelet cAMP phos-
phodiesterase (PDE) (2,23,38,46,65), are known to inhibit platelet aggregation (21,38,
40). Platelet aggregation is generally regarded as an important event in the initiation of
arterial thrombosis. The current interest in inhibitors of cAMP-PDE as therapeutic agents
has largely arisen from the recognition that there are distinct PDE isozyme families and
that tissues have different complements of isozymes.

Nomenclature and subcellular localization of cAMP-PDE isozymes are to be found in
articles from Beavo's (4,28) and Weishaar's laboratories (67,68). BMY20844 and
BMY21190 have the characteristics of selective type III inhibitors of "low Km" one of
which is high affinity for cAMP (38). The cAMP-PDE type III isozyme has been isolated
from or found in platelets (17,46), cytosolic fractions of bovine cardiac tissue (19), and

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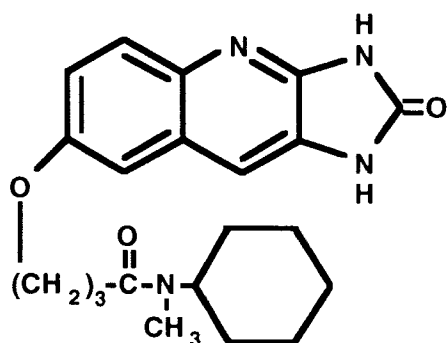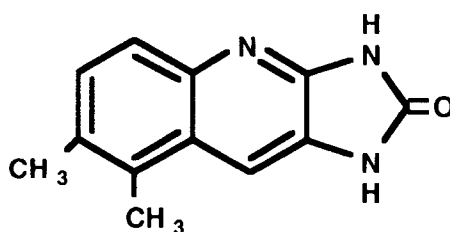
BMY 21190   BMY 20844

FIG. 1. The chemical structures of BMY20844 and BMY21190.

sarcoplasmic reticulum of canine heart muscle (30). This enzyme is also similar to the membrane-bound PDE in adipocytes (9), liver (73), and smooth muscle (36,69). The type III cAMP-PDE isozyme in the heart is the target of a number of agents that cause an increase in contractile force (18,24) and a decrease in peripheral vascular resistance in addition to inhibition of platelet aggregation (41).

The bipyridine cardiotonics, amrinone and milrinone, also inhibit cAMP-PDE (type III) and thereby the platelet aggregation. Studies in vitro and in vivo with amrinone have demonstrated impaired thromboxane A_2 formation (31,51,52,66). Aspirin and dipyridamole are not broad-spectrum inhibitors of aggregation. Anagrelide was a very promising candidate until its thrombocytopenic action was observed (1). Thus, in an attempt to develop other compounds possessing a favorable pharmacological profile, but lacking thrombocytopenic side effects, a series of related imidazoquinolines has been synthesized. While milrinone, a selective inhibitor of low Km cAMP-PDE, is a potent cardiac stimulant it did not produce thrombocytopenia in humans suggesting that the side effect was not related to inhibition of low Km cAMP-PDE (3). BMY21190 has a coronary vasodilating effect. There are no reports available with respect to the hemodynamic action of BMY21190. It was evaluated for its ability to modify the development of experimental thrombosis resulting from anodal current injury (100 μA for 6 hr) of the intimal surface of the left circumflex coronary artery (LCX) in anesthetized dogs (42). This review also summarizes the effect of BMY21190 on infarct size in a canine ischemic model that underwent a 90-min occlusion and 6-hr reperfusion of the left coronary artery (43).

CHEMISTRY

BMY20844 (1,3-dihydro-7,8-dimethyl-2H-imidazo [4,5-b] quinolin-2-one) and one of its derivatives, BMY21190 (7-[4-(N-cyclohexyl-N-methylamino)-4-oxobutoxy]-1,3-dihydro-2H-imidazo [4,5-b] quinolin-2-one) (Fig. 1) were synthesized by the Bristol-Myers Cardiovascular Chemistry Group, Wallingford, CT, USA. Compounds containing the imidazo [4,5-b] quinolin-2-one tricyclic nucleus are a new class of potent broad-spectrum inhibitors of platelet aggregation (40). These compounds are very insoluble in

water. The ring system of the imidazolin-2-one also exhibits a potent intrinsic activity that can be improved by introduction of substituents to the benzene ring. BMY20844, the prototype of these compounds, was identified as a promising clinical candidate due to its in vitro and in vivo potency for inhibition of platelet aggregation similar to analgrelide (40). Oral doses of BMY20844 provided protection similar to that of analgrelide in animal models of thrombosis. They produced, however, milder inodilating effects. BMY20844 is associated with much milder increases in ventricular contractile force and heart rate and with reductions in blood pressure in anesthetized ferrets and dogs (38). Rabbit heart cAMP-PDE isozymes were resolved by ion-exchange chromatography. BMY20844 inhibited only type III (a cGMP-inhibitable, low Km, cAMP-specific PDE) with an IC_{50} value of $5 \times 10^{-8} M$ (60). BMY20844 also inhibited canine cardiac sarcoplasmic reticulum membrane-bound cGMP-inhibitable, low Km, cAMP-specific PDE with virtually the same potency as the inhibition of cAMP-PDE in the platelet homogenate (60). In washed platelets, BMY20844 elevated cAMP levels and activated the platelet cAMP-dependent protein kinase. Introduction of functionalized side chains to this nucleus also markedly enhances biological activity, and BMY21190 inhibits ADP- and collagen-induced platelet aggregation in vitro with potency comparable to prostacyclin (IC_{50} values of 1–3 ng/ml). This compound has also been found to be a powerful inodilating agent following oral administration in dogs and ferrets, and has prompted a search for potent antithrombotic agents of this basic chemical structure (40).

ANTITHROMBOTIC EFFECTS OF BMY21190

Low Amperage Electrical Stimulation of Canine LCX Coronary Artery

BMY21190 was evaluated for its ability to modify the development of experimental thrombosis resulting from anodal current injury (100 μA for 6 hr) of the intimal surface of the left circumflex coronary artery (LCX) in anesthetized dogs (42). As shown in Fig. 2, distal to the arterial branch and proximal to its first descending branch, 1–2 cm of the LCX was isolated from the surrounding tissue by careful blunt dissection. Within this isolated region, an electromagnetic flow probe was affixed to the artery for the continuous measurement of LCX blood flow. To aid in the insertion of the LCX-stimulating electrode, the tip of a 25-gauge hypodermic needle was attached to a 28-gauge teflon-coated silver wire. This electrode was inserted through the wall of the LCX so that the tip of the electrode (2–3 mm) was in contact with the intimal lining of the vessel. The electrode was connected in series to a 250,000-ohm potentiometer, a 9-volt nickel-cadmium battery, and a digital ammeter (53). The circuit was completed by suturing a disc electrode to a subcutaneous region on the chest wall. The stimulating circuit was designed to permit constant monitoring and easy adjustment of the direct anodal current delivered to the intimal surface of the LCX. After basal hemodynamic measurements were recorded, constant infusion of BMY21190 (1 mg/kg over 30 min) or an equivalent volume of vehicle was delivered via the left jugular cannula. A constant infusion rate of 0.5 ml/min was maintained by an infusion pump. Thirty minutes after the start of the infusion, electrical stimulation of the LCX was initiated at 100 μA via the implanted silver wire electrode. Low amperage electrical stimulation of the canine LCX consistently produced occlusive coronary artery thrombosis (Fig. 2) (42,53).

Our model, in which intimal injury produced by low-amperage electrical stimulation of
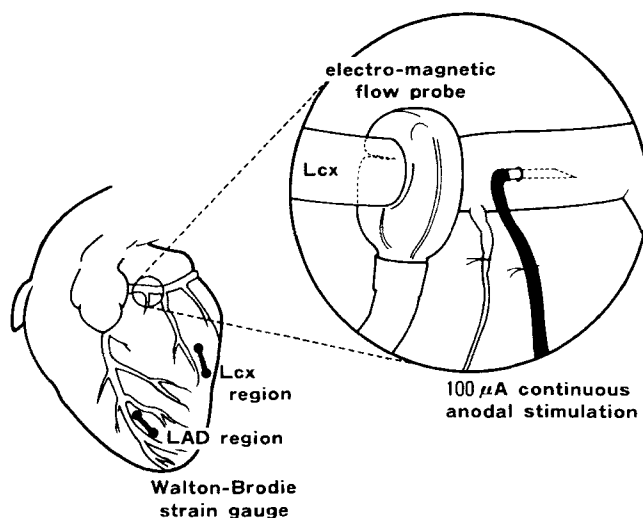
FIG. 2. Surgical procedure and anodal current stimulation in the canine heart. Adapted from ref. 42.

the LCX induces platelet adhesion and secondary recruitment of platelets to form aggregates, is a realistic simulation of coronary artery thrombus formation. The intimal platelet thrombus in our model is stabilized by the incorporation of fibrin into the growing thrombotic mass, and this developed thrombus shows morphological (42,53) and compositional characteristics similar to those of human coronary artery thrombi (10,44,53, 55). Thus, this method of inducing coronary artery thrombosis provides an appropriate model for studying the antithrombotic properties of compounds such as BMY21190.

BMY21190 or its vehicle was administered twice: before stimulation and 3 hr after stimulation (Fig. 3). Stimulation of the LCX was continued for 6 hr until LCX decreased to zero for at least 2 min (Fig. 3). The dogs were sacrificed by spontaneous or electrically-induced fibrillation. The thrombus present at the site of the electrical injury was removed from the intimal surface of the LCX and the thrombus wet weight was measured on an analytical balance. Values shown in this review are presented as mean ± SD.

Occlusive LCX thrombosis is determined primarily on the basis of total cessation (Fig. 3) of LCX blood flow (42). The time when thrombotic occlusion of the vessel occurs is readily determined by continuously monitoring the LCX blood flow. Complete cessation of the LCX blood flow occurred after approximately 3.2 hr of stimulation. However, signs of developed ischemia were evident as early as 45 min of the stimulation period. At this time, a reduction of the left ventricular isometric force in the LCX-perfused region of the myocardium, an alternation in the phasic coronary blood flow wave form, and a reduction in mean coronary blood flow were evidence that LCX thrombosis was occurring. After this, ST-segment elevation was recorded by the epicardial electrode situated in the LCX-dependent region of the myocardium. The left ventricular force in this region was further diminished. Occlusion and reperfusion of the coronary artery were repeated throughout the experiment. The earliest complete LCX occlusion by intravascular thrombosis occurred 75 min after the initiation of LCX coronary artery stimulation and terminated in ventricular fibrillation. Further elevation of the epicardial electrogram ST segments occurred as the LCX flow was reduced to zero. Isometric left ventricular contractile
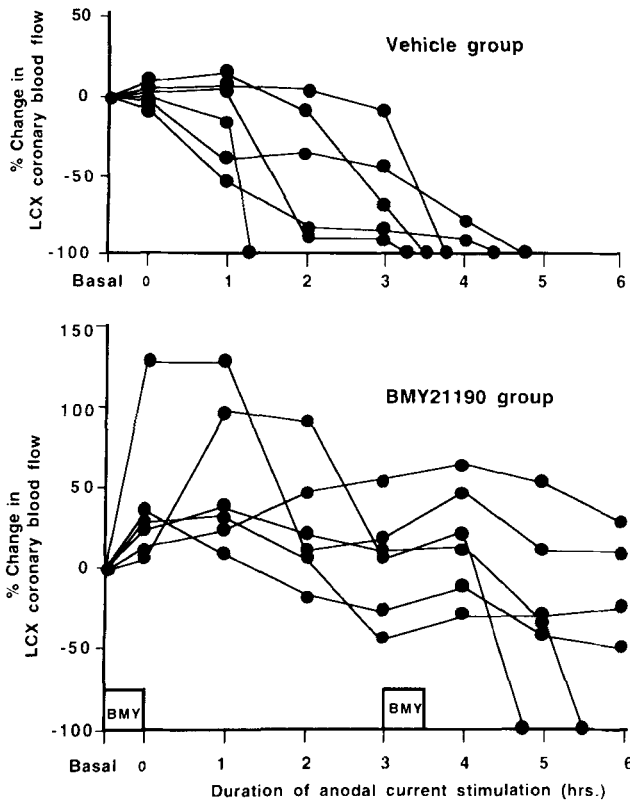
FIG. 3. Changes in left circumflex coronary artery blood flow for each dog in the vehicle and BMY21190 (1 mg/kg i.v.) treatment groups. Complete cessation of LCX blood flow as a result of thrombotic occlusion of the vessel is indicated by lines that intercept the abscissa. Lines that terminate before the 6-hr time point indicate spontaneous ventricular fibrillation. Adapted from ref. 42.

force was markedly reduced in both the ischemic (LCX) and the nonischemic regions. Finally, as indicated in Fig. 3, the LCX coronary blood flow of the control animals progressively declined over the course of the experiment until it completely ceased. Treatment of the animals with BMY21190 at a dose of 1 mg/kg i.v. prevented this decline. Thus, BMY21190 prolonged the life span of dogs in this coronary ischemic model. Furthermore, a second infusion of BMY21190 was able to dilate the coronary artery during the anodal current injury (42) (Fig. 3). Time to first hyperemia was observed significantly earlier in the vehicle as compared with the BMY21190-treated group during the LCX stimulation (Table 1). The average thrombotic mass that developed in response to intimal injury of the LCX coronary artery was significantly smaller in animals treated with BMY21190 than in the control vehicle dogs (Table 1). There was a 67% reduction in the incidence of occlusive LCX coronary thrombosis in the BMY21190-treated group (2 of 6 dogs occluded) as compared with the control vehicle group (6 of 6 dogs occluded) during the 6-hr stimulation period. The difference is significant (Fisher's Exact; p = 0.03).

Under the same experimental conditions, BMY20844 induced similar effects to those

TABLE 1. *Antithrombotic effect of BMY21190 on reactive hyperemic response and thrombus size in a canine model of coronary artery thrombosis*

	Vehicle (n = 6)	BMY21190 (1 mg/kg i.v.)(n = 6)
Time to first hyperemia (min)	142.0 ± 58.3	327.5 ± 50.6***
Time to occlusion (min)	217.5 ± 55.9	340.0 ± 40.0*
Ratio of experiments where at least one response occurred	6/6	2/6*
Average number of hyperemia episodes per experiment (n)	2.8 ± 6.3	0.7 ± 0.2
Average hyperemia magnitude (ml/min)	42.9 ± 31.4	12.5 ± 8.6
Thrombus size (mg)	19.8 ± 8.5	2.7 ± 0.4***

Mean ± SD.
*$p < 0.05$; *** $p < 0.001$ vs. vehicle.
Occlusive thrombi formation followed by spontaneous ventricular fibrillation occurred in all 6 of the 6 dogs in the vehicle group and in 2 of 6 dogs that received BMY21190. The other dogs in the BMY21190 group were sacrificed by electrical ventricular fibrillation at 360 min. The average time to complete thrombi occlusion of the LCX is included in the data of sacrificed dogs. Adapted from ref. 42.

of BMY21190 on time to first hyperemia and thrombus size in a dose-dependent manner (43) (Table 2). There was a 65% reduction in the incidence of occlusive LCX coronary thrombosis in the BMY20844-treated group (3 of 11 dogs occluded) as compared with the control vehicle group (7 of 9 dogs occluded).

Ex vivo Aggregation Study

Ex vivo aggregometry refers to experiments in which aggregometry was performed in vitro on platelet rich plasma (PRP) samples obtained from dogs dosed with BMY21190 (1 mg/kg). Platelet reactivity was assessed in vitro using PRP obtained from venous blood collected in 3.8% citrate (1 ml/10 ml) before stimulation (prestimulation) and 4 hr after stimulation (poststimulation). The blood was centrifuged at 380 × *g* for 5 min to obtain the PRP and again at 2200 × *g* for 10 min to yield platelet poor plasma (PPP). The PRP was adjusted to 200,000 platelets/μl by adding PPP. Aggregation responses were measured within 15 min of PRP preparation using a PAP-3 platelet aggregometer (Bio Data Corp., Willow Grove, PA) to record the percentage of change in the light transmission of

TABLE 2. *Antithrombotic effect of BMY20844 on reactive hyperemic response and thrombus size in a canine model of coronary artery thrombosis*

	Vehicle	BMY20844	
		1 mg/kg i.v.	3 mg/kg i.v.
Time to first hyperemia (min)	106.1 ± 63.4 (n = 9)	256.0 ± 127.2* (n = 5)	302.0 ± 60.8*** (n = 6)
Thrombus size (mg)	22.5 ± 9.0 (n = 9)	10.3 ± 3.2* (n = 5)	5.4 ± 8.9* (n = 6)

Mean ± SD.
* $p < 0.05$; *** $p < .001$ vs. vehicle.
Adapted from ref. 44.

PRP (set at 0%) relative to PPP (set at 100%). Aggregation was initiated by adding 50 μl of either ADP (23.4, 58.5, or 117 μM) (Sigma Chemical Co., St. Louis, MO), collagen (1/100, 1/800, or 1/1600) (Ethicon Collagen Dispersion), or arachidonic acid (0.325 or 0.65 mM) (Sigma Chemical Co., St. Louis, MO) to 450 μl of PRP. A total of eight different concentrations of three compounds was tested. Posttreatment responses for each animal were compared with the prestimulation responses. The data are shown in Table 3. Ex vivo platelet aggregation in response to ADP, arachidonic acid, and collagen was significantly inhibited by BMY21190. There were no significant changes in the platelet counts after administration of BMY21190.

Infused at a dose of 1 mg/kg BMY21190 inhibited ex vivo platelet aggregation and prevented thrombotic occlusion of the LCX at the site of the electrically induced intimal injury. Postmortem examination of the LCX coronary artery revealed that in dogs whose LCX coronary blood flow had stopped, intravascular thrombi completely filled the arterial lumen. This progressive decline and ultimate cessation of LCX blood flow correlate with the complete thrombotic filling of the vascular lumen. The vasodilating properties of BMY21190 may play a role in maintaining the LCX blood flow by relaxing vascular tone and permitting blood to flow between the arterial wall and the thrombotic mass.

Under the same conditions, ex vivo platelet aggregation in response to arachdonic acid was inhibited significantly by BMY20844 (44). No significant changes were demonstrated in the platelet counts after administration of BMY20844 (1 mg/kg i.v.). In rats significant inhibition of collagen- and/or ADP-induced aggregation by BMY20844 was observed ex vivo two hr after oral administration in a water suspension (8).

HEMODYNAMIC EFFECTS OF BMY21190

Effects of BMY21190 in a Canine Model of Coronary Artery Thrombosis

Hemodynamic data were recorded continuously during the course of the experiment on a six-channel Grass polygraph. Arterial blood pressure transducer was attached to a polyethylene catheter that was placed in the ascending aorta via the left carotid artery. Segmental left ventricular isometric contractile force in the regions of the myocardium which depend on the LCX and the left anterior descending coronary artery (LAD) was measured with a Brodie-Walton strain gauge arch.

A summary of the hemodynamic effects of BMY21190 after 30 min of infusion is

TABLE 3. *Effects of BMY21190 (1.0 mg/kg i.v.) on ex vivo aggregation in a canine model of coronary artery thrombosis*

	Vehicle (n = 8)		BMY21190 (n = 6)	
	pre	post	pre	post
Platelet aggregation (%)				
ADP (58.5 μM)	50.6 ± 20.1	49.3 ± 17.5	54.3 ± 27.7	22.5 ± 12.5*
AA (0.65 mM)	51.0 ± 30.5	54.0 ± 30.5	66.0 ± 37.7	8.8 ± 7.6*
Collagen (1:100)	61.1 ± 8.2	63.0 ± 18.7	72.0 ± 17.4	23.8 ± 23.7*

Mean ± SD.
* $p < 0.05$ per v. post.
AA: arachidonic acid.
Adapted from ref. 42.

shown in Table 4. BMY21190 exhibits a pronounced antithrombotic effect when admin-
istered at a dose of 1 mg/kg i.v. Table 4 also indicates the marked hemodynamic effects
of BMY21190. Although no significant changes were observed in the double product or
tension, a reflex elevation of heart rate was demonstrated in the BMY21190-treated
animals. The largest reduction in MAP occurred in the BMY21190 (1 mg/kg) group
where, after 30 min of infusion, the MAP was reduced by 67% compared with the basal
reading. After the first hyperemia, the LCX blood flow steadily declined to complete
cessation in both control and BMY21190 groups. All control animals developed occlusive
thrombi. BMY21190, infused at a dose of 1 mg/kg, prevented the decline and eventual
cessation of LCX blood flow characteristic in the control group. The left ventricular
isometric force of the control dogs also declined slightly over the course of the experi-
ment. The BMY21190-induced alterations in the decline of the left ventricular force were
relatively small. A second infusion of BMY21190 after thrombotic occlusion of the LCX
increased coronary blood flow. There also were marked differences between the
BMY21190-treated and control animals with respect to intravascular thrombotic mass.
These findings suggest that BMY21190 prevents occlusion of the LCX primarily by
inhibiting thrombus formation at the site of intimal injury.

The hemodynamic effects of BMY21190 are similar to those of other cAMP-PDE
inhibitors. BMY21190 is a vasodilator which produces a reduction in arterial blood
pressure and an increase in heart rate. BMY21190 and other closely related compounds

TABLE 4. *Hemodynamic effects of BMY21190 in a canine model of coronary*
artery thrombosis

	Vehicle (n = 9)	BMY21190 (1 mg/kg i.v.)(n = 6)
Heart Rate (bpm)		
before	156.2 ± 6.5	157.6 ± 5.3
after	161.1 ± 8.0	215.3 ± 11.6
P	ns	P < 0.001
Mean AP (mm Hg)		
before	104.8 ± 12.9	108.6 ± 7.3
after	106.2 ± 4.1	72.5 ± 4.5
P	ns	P < 0.001
Mean CBF (ml/min)		
before	26.4 ± 2.2	31.8 ± 7.2
after	26.2 ± 2.1	40.5 ± 8.9
P	ns	P < 0.02
Tension-LAD (g)		
before	74.5 ± 11.3	71.6 ± 9.7
after	80. ± 12.8	78.3 ± 12.9
P	ns	ns
Tension-LCX (g)		
before	74.7 ± 6.1	62.2 ± 6.3
after	78.3 ± 6.5	64.6 ± 8.6
P	ns	ns

Mean ± SD.
After = 30 min after drug administration.
AP: arterial pressure; CBF: coronary blood flow; LAD: left anterior descending coronary artery; LCX: left
circumflex coronary artery.
Adapted from ref. 42.

have been shown to significantly inhibit platelet cAMP-PDE at low nanomolar doses (40). With systemic infusion at a dose of 1 mg/kg BMY21190 resulted in a marked inhibition of platelet activation measured ex vivo, with no significant change in ventricular contractile force. These pharmacological actions appeared to be the effect of a direct action on cardiac and vascular smooth muscle and were generally seen at doses higher than those required to produce significant effects on platelet aggregation (12,14,20). Amrinone and milrinone inhibit platelet activation in vitro with an efficiency that parallels their abilities to inhibit platelet cAMP-PDE and to increase myocardial contractile force (5,62,64). On the other hand, administration of BMY21190 did not cause any significant positive inotropic effect in the present canine model of coronary artery thrombosis.

Effects of Intracoronary Administration of BMY21190 on the Coronary Blood Flow and Oxygen Content

In order to elucidate the direct effect of BMY21190 on regional tension development and coronary blood flow and oxygen content, BMY21190 was administered directly into the coronary circulation in the canine heart (42). Male mongrel dogs were anesthetized with dial urethane (0.6 mg/kg i.v.) and ventilated under positive pressure with room air using a Harvard respirator pump. Tidal volume and respiratory rate were adjusted to maintain blood pH, PO_2, and PCO_2 within normal limits (Instrument Lab., Blood Gas Analyzer, Micro 13). Distal to the first diagonal branch, 1 cm of the LAD was isolated from surrounding tissue by careful blunt dissection. Within this isolated region, an electromagnetic flow probe was affixed. An intracoronary catheter for the drug infusion was inserted through the vessel wall of the LAD at a distance 3 mm distal to the electromagnetic flow probe. Patency of the intracoronary catheter was maintained throughout the experiment by infusing a 0.9% saline solution at a rate of 0.3 ml/min. A segment of the great coronary vein was also isolated near the LAD area. An intracoronary venous catheter was placed in the great vein draining the LAD region to sample coronary venous blood. Another tube was inserted into the left atrial appendage (LA) for the withdrawal of arterial blood. Atrial pacing was performed by a Grass model S-44 stimulator and a Grass model PSIU-6 isolation unit. Propranolol (0.5 ml/kg i.v.) was administered slowly to maintain a constant heart rate. BMY21190 or vehicle was rapidly injected into the coronary artery in 0.2 ml. Blood was periodically drawn from the LAD and LA. Atrial and venous oxygen contents were determined before each dose of BMY21190.

As shown in Table 5, BMY21190 (0.01 and 0.03 mg/kg i.c.) increased the coronary blood flow significantly. The oxygen content of the left atrial appendage (A) did not change after BMY21190 (0.01 mg/kg i.c.) administration, but the oxygen content of the great coronary vein (V) in the BMY21190-distributed area increased significantly. BMY21190 (0.01 mg/kg i.c.) reduced the oxygen A-V difference significantly (Table 6). BMY21190 also caused significant coronary vasodilation without increasing myocardial oxygen consumption.

As shown in Fig. 4 isometric tension in the area of the LAD was significantly increased after BMY21190 (0.01 mg/kg i.c.) administration. A large dose of BMY21190 (0.1 mg/kg i.c.), however, produced a significant negative inotropic effect. This negative inotropic effect was significantly reversed by verapamil (0.5 mg/kg i.v.). Diltiazem (0.5 mg/kg i.v.), however, did not affect this negative inotropism. In voltage clamp studies, milrinone (64), and amrinone (37,64) increased the amplitude of I_{ca}. These agents pro-

TABLE 5. *Mean coronary blood flow change after intracoronary injection of BMY21190 in a*
propranolol-treated canine constant heart rate model with atrial pacing

	Changes of mean coronary blood flow (LAD; ml/min)	
	Before	After BMY21190
BMY21190 (mg/kg)		
0.01 (n = 10)	29.9 ± 9.5	52.8 ± 19.9*
0.03 (n = 9)	38.1 ± 16.2	62.9 ± 24.8***
0.1 (n = 10)	4.14 ± 16.7	55.8 ± 28.5

Mean ± SD.
* $p < 0.05$; *** $p < 0.001$ vs. before.
BMY21190 was given by rapid intracoronary injection in a volume of 0.2 ml containing the above amounts of drug.
Adapted from ref. 42.

duced a positive inotropic effect and increased ^{45}Ca uptake that is sensitive to verapamil (50). These results suggest that a large dose of BMY21190 induces an excessive calcium accumulation in the myocardial cells.

Effect of BMY21190 on Coronary Artery Occlusion and Reperfusion

Ischemic myocardial injury was produced in dogs by means of techniques detailed in previous publications (55). Male mongrel dogs were anesthetized with dial urethane, intubated, and ventilated with room air using a Harvard respirator. The LCX was isolated just before the first diagonal branch. LCX coronary blood flow was measured by placing an electromagnetic flow probe (Carolina Medical Electronics, Inc.) distal to an occlusive ligature. To limit reperfusion arrhythmias and myocardial hemorrhage, the occlusive ligature was adjusted so that basal coronary blood flow was not altered. After measurement of basal coronary blood flow, stenosis of the artery was produced by affixing a silk ligature around both the vessel and an 18- or 19-gauge needle. The degree of partial constriction was adjusted to reduce by at least 70% the hyperemic response to a 10-sec occlusion without altering basal flow. Five minutes later, myocardial ischemia was produced by clamping the LCX flow completely for a period of 90 min. The mechanical

TABLE 6. *Effect of intracoronary (LAD) administration of BMY21190 on the oxygen content of*
canine left atrial appendage and great coronary venous blood

	Pre-drug (n = 6)	Post BMY21190 (0.01 mg/kg i.c.)(n = 6)
Oxygen content (%)		
LA(A)	16.3 ± 3.1	15.4 ± 5.4
Great vein (V)	4.2 ± 1.1	7.8 ± 3.6*
A-V	12.5 ± 2.2	7.7 ± 2.6*

Mean ± SD.
* $p < 0.05$ vs. pre-drug.
LA: left atrium; A-V: A-V difference.
Adapted from ref. 42.

RApacing(135/min)   dog:20.0kg
200 ── BMY21190(0.01mg/kg.i.c.) ──

BP 100
(mmHg)

0

50
Tension
(LCX)
(g)   0

50
Tension
(LAD)
(g)   0

50
pulsatile
CBF
(ml/min)0

50
mCBF
(ml/min)
0   1min
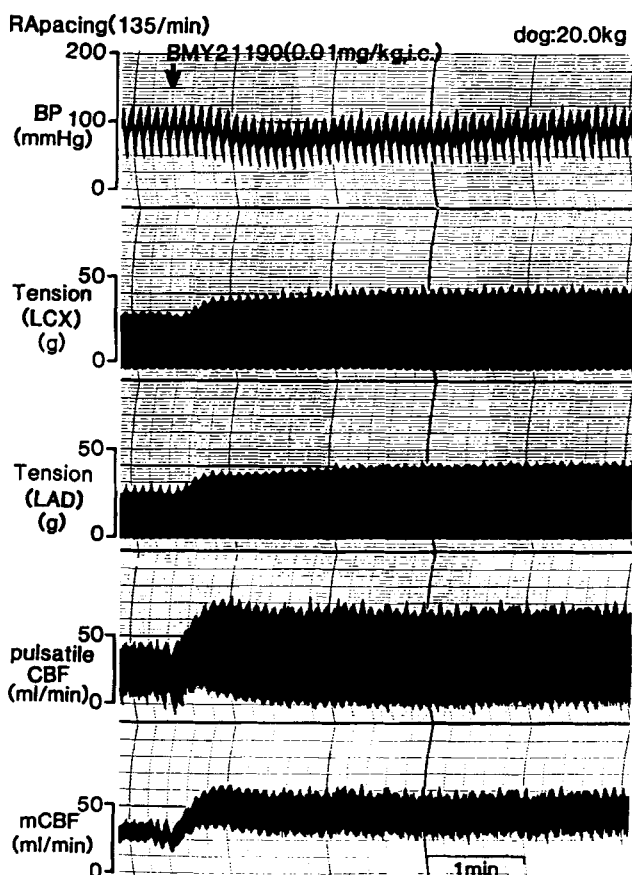
FIG. 4. The effect of BMY21190 (0.01 mg/kg i.c.) on various cardiovascular parameters in the canine constant heart rate model. Adapted from ref. 42.

occlusion was released gradually over a period of 30 min to prevent reperfusion induced ventricular fibrillation. Dogs remained anesthetized until sacrificed by electrical fibrillation after the 6-hr reperfusion period. The experimental protocol consisted of a 90-min occlusion and 6-hr reperfusion of the LCX.

While there is little doubt that platelets accumulate within infarcted myocardium (45,56), the role of platelets in the development of myocardial infarction is unclear. Platelets have been shown to form microemboli in ischemic myocardium (45) and to release vasoactive substances, including TXA_2 (25,63). Reperfusion produces a 10-fold increase in platelet accumulation, as compared to that found with permanent occlusion (33,53). BMY21190 induced a significantly higher heart rate and lower mean arterial blood pressure than observed in the vehicle group. In general, the increase in pressure-rate products is an unfavorable action of cAMP-PDE inhibitors in patients with ischemic heart disease. Although BMY21190 induced a rise in heart rate, it had no significant effect on the pressure-rate products. Mean LCX coronary blood flow after reperfusion was significantly less than the preocclusion flow in the vehicle group. BMY21190 administration, however, maintained coronary blood flow after reperfusion. There was a significant

difference between the vehicle and the BMY21190 groups with respect to mean coronary blood flow after reperfusion.

REDUCTION OF ISCHEMIC MYOCARDIAL INJURY

Postmortem Quantification of Infarct Size

After a 90-min occlusion and 6-hr reperfusion of the LCX, myocardial infarct size was quantified by an in vitro dual perfusion technique previously described (55). Cannulae were inserted into the LCX immediately distal to the site of LCX occlusion and into the aorta above the coronary ostia. The LCX coronary bed was perfused with 1.5% triphenyl tetrazolium hydrochloride (TTC) in 20 mM potassium phosphate buffer (pH 7.4, 37°C). The aorta was perfused in a retrograde manner with 0.25% Evans blue dye. Both the LCX region and the remainder of the heart were simultaneously infused with their respective stains at a constant positive pressure of 100 mmHg for 5 min in a water bath (37°C). The stained heart was then sliced into six or seven sections, approximately 1.0-cm thick, from apex to base. The area free of the blue dye indicated the area of the left ventricle at risk of infarction. The region of infarcted myocardium within the area at risk was demonstrated by the lack of staining of the tissue when perfused with TTC due to a loss of dehydrogenase enzymes which convert the colorless TTC to a red formazan precipitate.

Analysis of Infarct Size

More specifically, the upper surface of the transverse ventricular sections were traced carefully onto clear plastic sheets. In order to provide a permanent record of infarct morphology, the areas of each zone were measured by planimetry using a graphic tablet and Apple IIe computer (Apple Computer, Inc., Cupertino, CA, USA). A custom-made software program was used to calculate the masses of the infarct zone and the area at risk from the planimetered areas and the weights of each transverse ventricular section. Infarct size was calculated as a percentage of both the area at risk and the total left ventricle. Previous studies demonstrated that there is an excellent correlation between infarct size derived by the planimetric method and the direct gravimetric measurement (70). The measurement of infarct size using the TTC technique also correlated with the infarct size measured by histological examination. Transmural tissue blocks from the area at risk were obtained from three transverse sections of the hearts.

There is increasing evidence that activated neutrophils cause myocardial injury during the reperfusion of ischemic myocardium (35,72). Reperfusion of ischemic myocardium may have both beneficial and detrimental effects on ischemic cells (7). Myocardial infarction expands with the duration of coronary occlusion (29). Moreover, infarct size may be increased by reperfusion. After occlusion and reperfusion, the infiltration of neutrophils into ischemic myocardium could exacerbate and promote myocardial damage (71). The invading neutrophils may injure the myocardial vasculature by generating oxygen free radicals (47,54). While BMY20844 and BMY21190 increase the concentration of cAMP in leucocytes (38,43), free radical production is inhibited by the agents that produce cAMP accumulation (34). The increase in leukocyte cAMP will inhibit the activation of oxidative burst and chemotaxis.

In order to elucidate the effect on infarct size, BMY21190 was administered before

reperfusion of the ischemic dog heart. Two dogs failed to complete the protocol due to intractable ventricular fibrillation after reperfusion. Thus, infarct size data are reported for six dogs treated with BMY21190 and for six dogs treated with vehicle (Table 7). As demonstrated previously (71), the gravimetric and planimetric methods of quantifying infarct size were in excellent agreement. For infarct size expressed as a percentage of the area at risk, the equation of liner regression was: planimetric = 1.05 (gravimetric) + 0.08 (r = 0.99, SE = 2.43, n = 14). The data of infarct zone weight, risk zone weight, the ratio of infarct zone/left ventricular mass (LV), and area at risk are summarized in Table 7. Total LV mass and risk weight of BMY21190-treated dogs tended to decrease compared with those of vehicle-treated dogs. No significant difference, however, was demonstrated between them. Also, there was no significant difference between the area at risk/LV in BMY21190-treated dogs and that in vehicle-treated dogs. As shown in Table 7, in the control dogs (vehicle), mean infarct size was 62.4 ± 9.6% of the anatomic area at risk. In dogs receiving BMY21190, infarct size averaged 36.1 ± 17.6% of the area at risk. Infarct size was significantly different from controls, whether expressed as grams of infarcted tissue or percentages of the area at risk (Table 7).

Relationship Between Infarct Size and Myeloperoxidase Activity

Because neutrophil infiltration is heterogenous (48), the transmural distribution of myeloperoxidase (MPO) activity across the ischemic region was studied in segments of tissue obtained from the infarct and risk zones. In addition, we examined the correlation between infarct size and MPO activity in a canine model undergoing a 90-min occlusion and 6-hr reperfusion. Bradley et al. (6) have utilized a spectrophotometric assay for the neutrophil-specific MPO enzyme to measure neutrophil accumulation in the ischemic myocardium. MPO serves as a quantitative indicator of neutrophil infiltration (48). MPO, contained within the azurophilic granules of the neutrophils, was liberated as follows. Myocardial tissue was frozen in liquid nitrogen and pulverized. Samples of myocardium

TABLE 7. *Effect of BMY21190 on canine infarct size after a 90-min occlusion and 6-hr reperfusion of the left circumflex coronary artery*

	Vehicle (n = 6)	BMY21190 (1 mg/kg i.v.)(n = 6)
Gravimetric method		
Body weight (kg)	15.4 ± 0.9	14.0 ± 1.3
Critical stenosis (%)	71.3 ± 7.2	60.6 ± 6.0*
LV weight (g)	93.4 ± 13.4	79.1 ± 39.3
Area risk weight (g)	42.8 ± 7.6	34.9 ± 8.0
Infarct zone weight (g)	24.1 ± 6.7	13.0 ± 7.2*
Planimetric method		
Area risk/LV (%)	42.3 ± 11.8	44.4 ± 6.3
Infarct zone/LV (%)	27.4 ± 11.4	16.1 ± 7.8
Infarct zone/area risk (%)	62.4 ± 9.6	36.1 ± 17.6*

Mean ± SD.
*$p < 0.05$ vs. vehicle.
The quantification of the masses of the infarct zone, area at risk and left ventricle was done by both gravimetric and planimetric methods.
Adapted from ref. 43.

(50–200 mg) were taken from the central infarct region, the noninfarcted tissue within the area at risk, and the overlapping border between the infarct region and the area at risk. Tissue was homogenized at 4°C in 0.5% hexadecyltrimethyl ammonium bromide (HTAB, Sigma Chemical Co.) buffered with 50 mM potassium phosphate (pH 6.0) with a polytron homogenizer (Kinematica Ag, Littau/Lucerne, Switzerland). The tissue suspensions were then sonicated for 10 sec on ice with a probe sonicator (Heat Systems-Ultrasonics, Inc., New York, NY, USA Model W-375). These samples were then centrifuged (20,000 g for 15 min) and the supernatants were assayed for MPO activity. MPO was assayed spectrophotometrically by adding 0.05 ml supernatant to 1.45 ml 50 mM potassium phosphate buffer (pH 6.0) with 0.167 mg/ml o-dianisidine hydrochloride (Sigma Chemical Co.) and 0.0005% hydrogen peroxide (Sigma Chemical Co.). The change in absorbency was measured at 460 nm with a Cary 210 Spectrophotometer (Cary Instruments, Monrovia, CA, USA). One unit of MPO (MPO/100 mg tissue weight) activity was diluted to the amount of enzyme required to degrade 1 μmol hydrogen peroxide per minute at 25°C (48).

As shown in Fig. 5, the highest MPO activity was found at the risk area where the activity at 6 hr was 0.40 U MPO/100 mg tissue. Activity in the central infarct zone was 0.30 U MPO/100 mg tissue. MPO activity (0.14 U/100 mg tissue) obtained from the area at risk treated by BMY (1 mg/kg i.v.), however, was significantly lower than that of the vehicle treated tissue (p < 0.05). The MPO activity in the infarct zone obtained from both groups was almost the same value suggesting that BMY21190 may be effective only on the area at risk. The relationship between MPO activity and infarct size was studied in 12 dogs subjected to a 90-min occlusion and 6-hr reperfusion. Six of the infarcted dogs
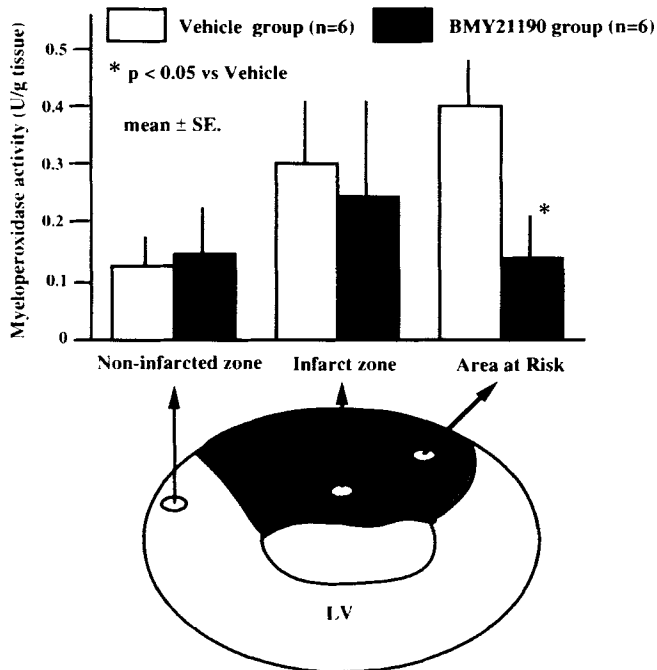

FIG. 5. Effects of BMY21190 on tissue myeloperoxidase activity in a canine model of occlusion and reperfusion. Adapted from ref. 43.

pretreated with BMY21190 (1 mg/kg i.v.) were also included. The values show the infarct size (ratio area at risk) and the MPO activity obtained from the zone of area at risk. The ratio correlated well with the MPO activity (r = 0.683, p < 0.02). The relation was y = 2.95 x + 11.5, including both groups (n = 12).

Infarcted myocardium from BMY21190-treated dogs showed significantly lower MPO activity than that of vehicle-treated dogs. MPO activity in the area at risk treated with BMY21190 was significantly lower than that in vehicle-treated tissue. However, the MPO activity in the infarct zone obtained from both groups was almost the same. According to the protocol, BMY21190 was injected 45 min after the occlusion to prevent reperfusion injury. The infarction produced by this protocol may occur in two steps: first, it may occur simply through occlusion (13 g); secondly, it may develop through reperfusion injury (13 to 24 g) induced by neutrophil infiltration. BMY21190 may be effective on the area at risk. In this protocol, BMY21190 does not affect the first step of infarct. For these reasons, the MPO activity in the infarct zone obtained from both groups might have been almost the same.

INOTROPIC AND CHRONOTROPIC EFFECT OF BMY21190 IN ISOLATED RABBIT PAPILLARY MUSCLES AND ATRIAL PREPARATIONS

BMY21190 was studied for its inotropic and chronotropic actions on rabbit isolated heart muscle preparations. At concentrations between 10^{-7} and 10^{-5} M, BMY21190 showed a mild but significant positive inotropic effect (p < 0.05) of 113.6 ± 11.8% (10^{-6} M) (n = 9) as compared with the vehicle control. Time-to-peak tension was decreased in a concentration-dependent manner (not significant). At a concentration of 10^{-4} M, BMY21190 reduced the contractile force: 80.7 ± 9.2% (p < 0.01 vs. vehicle group). In an in vivo study in large doses BMY21190 also induced a significant negative inotropic effect (42). This negative inotropic effect was partially reversed by verapamil. BMY21190 did not exert a positive chronotropic effect at concentrations between 10^{-7} to 10^{-5} M on rabbit isolated atrial preparations. In an in vivo study BMY21190 produced an increase in heart rate. BMY21190 caused a reduction in arterial blood pressure. The change of heart rate induced by BMY21190, therefore, may be a reflex tachycardia. Thus, BMY20844 and BMY21190 possess antithrombotic and moderate inotropic actions. In isolated cardiac tissues, PDE inhibitors exert a positive inotropic effect which is probably mediated by cAMP (11,49). This suggests that PDE III is the molecular target for some cardiotonic drugs (19).

The effect of inotropic therapy on myocardial infarct size was not completely evaluated. Previous investigators have demonstrated, however, that dobutamine does not increase infarct size in humans (16) or dogs (32). Further studies are needed concerning the effects similar to those of dobutamine.

It is suggested that the new inotropic vasodilator BMY21190 may have beneficial hemodynamic effects in patients with congestive heart failure. In numerous studies milrinone and enoximone have clearly been shown to improve hemodynamics in patients with congestive heart failure (27). The strength of milrinone and other type III PDE inhibitors as positive inotropes and vasorelaxants parallels their potency as cardiac and vascular type III PDE inhibitors (61). These data support the concept that a single mechanism of action may account for the cardiotonic and vasodilator efficacy of milrinone and other type III PDE isozyme inhibitors (11).

MYOCARDIAL cAMP LEVELS

The precise mechanism by which BMY21190 induces neutrophil infiltration is unknown. The type III cAMP-PDE isozyme in the heart is the target of several agents (11) that increase contractile force (18) and decrease peripheral vascular resistance, as well as inhibit platelet aggregation. These agents produce an increase in cardiac inotropy and in ^{45}Ca uptake (37,50,64). The activation of oxidative metabolism is reported to be modulated by cyclic nucleotides and cAMP-accumulating agents (34). Free radical production is inhibited by exogenously added cAMP and agents which increase the levels of intracellular cAMP (34).

We determined the level of myocardial cAMP content in the isolated rabbit heart. The hearts of male New Zealand white rabbits (1.0–1.5 kg) were quickly isolated. Rabbit heart muscles were placed in contact with platinum electrodes and stimulated using a Grass stimulator (S88) set. After incubation, the experiments were performed as follows; the procedure was based on that as described by Sabina et al. (57). Prior to drug (or vehicle) addition and 10 min after the final concentration of BMY20844 and BMY21190, the heart muscle preparations were removed from the organ bath. The specimens were plunged and pressed between the jaws of "quick-freeze tongs," which had been cooled within 5 sec using liquid nitrogen. The samples were ground to a fine powder with a mortar and pestle in an ice bucket surrounded by dry ice in acetone. The powder was centrifuged at 10,000 × 4°C for 15 min. To aliquots of 100 μl of supernatant, 1 ml of water-saturated ether was added and the mixtures were centrifuged under the same conditions. Ether extraction was repeated three more times. After this procedure, the aliquots of supernatant were boiled for 1 min. Radioimmunoassay of tissue cAMP (26) was carried out after acetylation using triethylamine and acetic anhydride. As shown in Table 8, both BMY20844 (10^{-6} M) and BMY21190 (10^{-6} M) significantly increased myocardial cAMP content compared with that of the vehicle-treated rabbit heart. These drugs have a significant positive inotropic effect on isolated rabbit papillary muscle, suggesting a relationship between the positive inotropic action and the tissue level of cAMP. BMY21190 induces both cAMP and calcium accumulation in the myocardial cells and may protect them from exposure to oxygen radicals.

SUMMARY

Compounds containing the imidazoquinoline nucleus are a new class of potent broad-spectrum inhibitors of platelet aggregation (38). BMY21190 is an ether-linked side chain derivative with a simply substituted imidazoquinoline. These compounds are potent inhibitors of platelet cAMP PDE (IC_{50} $10^{-8}M$) but have little effect on platelet homogenate cGMP PDE (IC_{50} > $10^{-5}M$) (8). BMY21190 was evaluated for its ability to modify the development of experimental thrombosis resulting from anodal current injury (100 μA for 6 hr) to the intimal surface of the left circumflex coronary artery (LCX) in the anesthetized dog (42). After a 30-min administration of BMY21190, heart rate and mean coronary blood flow were increased significantly and mean arterial pressure was decreased. The myocardial tension of the LAD and LCX areas, however, did not change significantly after BMY21190 infusion. During LCX stimulation, the first LCX occlusion and the hyperemic reaction occurred significantly earlier in the control group than in the BMY21190 group. Compared with the controls, BMY21190 treatment reduced the de-

TABLE 8. *Myocardial cAMP levels of BMY20844- and BMY21190-treated rabbit heart*

	Vehicle (n = 8)	BMY20844 $10^{-6} M$ (n = 7)	BMY21190 $10^{-6} M$ (n = 7)
cAMP contents (p moles/mg tissue)			
Right ventricle	0.524 ± 0.190	0.793 ± 0.191*	0.952 ± 0.315*
Left ventricle	0.314 ± 0.166	0.545 ± 0.423	0.911 ± 0.276**

Mean $\pm$ SD.
* $p < 0.05$, ** $p < 0.01$ vs. vehicle.
n = numbers of rabbits.
Dose ($10^{-6} M$) shows the final concentration in the organ bath (Minami, Driscol and Lucchesi, unpublished data).

velopment of LCX thrombus mass. In ex vivo studies, platelet aggregation in response to arachidonic acid, ADP, or collagen was inhibited by BMY21190.

The precise mechanism by which BMY21190 induces platelet inhibition is unknown. Agents capable of increasing intraplatelet accumulation of cAMP effectively inhibit platelet aggregation induced by broad-spectrum agonists (8,41,60). Elevation of platelet cAMP leads to phosphorylation of platelet myosin light chain kinase by a cAMP-dependent protein kinase (22). The cAMP-mediated phosphorylated and inactivated form of myosin reduces actin-myosin interaction, platelet contraction, and granule secretion (22).

The effect of BMY21190 on infarct size was also evaluated using a canine ischemic model with 90-min occlusion and 6-hr reperfusion of the left coronary artery (42). The infarct zone/area at risk of the BMY21190 group was significantly smaller than that of the vehicle group. Myeloperoxidase activity, an indicator of neutrophil infiltration, measured in the area at risk from hearts of the BMY21190-treated group was significantly lower than that in the vehicle treated tissue. BMY21190 may reduce the infarct size partly through the inhibition of neutrophil infiltration in the canine model. BMY21190 achieved a desired antithrombotic effect without thrombocytopenic side effects. In addition, the strong coronary vasodilating and mild inotropic actions of BMY21190 had a significant impact on cardiac hemodynamics. BMY21190 and BMY20844 represent the first compounds of this series to be selected for clinical testing (40). BMY20844 was selected from this series as a candidate for clinical assessment and has been studied in some detail at the biochemical (60) and pharmacological levels (8).

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