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Stereoselective Effects of *cis*- and *trans*-Cyclopropylbis(dioxopiperazines) Related to ICRF-159 on Metastases of a Hamster Lung Adenocarcinoma¹

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The synthesis for cis-4,4'-(1,2-cyclopropanediyl)bis(2,6-piperazinedione) (cis-3) is discussed. Stereoselective effects on metastases of cis-3 and the previously reported trans-2 isomer were compared to conformationally mobile ICRF-159 using a Syrian hamster lung adenocarcinoma (LG1002). Whereas ICRF-159 and cis-3 significantly inhibited lung metastases, the trans-2 isomer significantly increased the number of metastatic nodules in the lung. Thus, these studies have revealed that, at least in one tumor model, antimetastatic activity can be separated from metastatic potentiating activity by controlling drug geometry.

A previous report from these laboratories described the comparative effects of ICRF-159 (1), the *trans*-cyclopropyl



analogue 2, and certain related tetraacids and esters on cytotoxicity, mutagenicity, and scheduled and unscheduled DNA synthesis in tissue culture.² However, perhaps of greater significance is the observation that ICRF-159 inhibits metastases in the Lewis lung tumor (3LL) animal model without impeding the growth of the primary implant.³⁻¹³ Histological examination of blood, lungs, and primary tumors indicated that antimetastatic activity is likely due to normalization of the developing blood vessels at the invading margins of the primary tumors.^{4,9} Whereas this angiometamorphic effect is not unique to 3LL infected animals,^{8,14,15} histological features suggested that ICRF-159 antimetastatic effects in an experimental transplanted murine squamous carcinoma did not depend upon morphological changes in vascularity.¹⁶ Although ICRF-159 does not reduce metastases in all tumor models,¹⁷ it is particularly interesting to note the results of Lazo et al.¹⁸ These investigators have observed that incubation of exponentially growing B16 melanoma cells with ICRF-159 significantly increased their in vivo lung colony-forming efficiency.¹⁸ Concurrently, we have been investigating the

antimetastatic effects of ICRF-159 and the *trans*- and *cis*-cyclopropyl analogues (2 and 3, respectively) in the allogeneic hamster lung adenocarcinoma model. A priori we discuss the synthesis of *cis*-3 and our preliminary biological results revealing the stereoselective actions of 2 and 3 on metastases in this animal model.

Synthetic Aspects. The synthesis for *cis*-3 from *cis*-1,2-cyclopropanedicarboxylic acid (4) is similar, but not



identical, to the reported² preparation of *trans-2* from the corresponding *trans*-dicarboxylic acid. Starting *cis-4* was prepared according to the method of Payne^{19a} and McCoy^{19b} and readily converted to the diacid chloride **5** by treatment with PCl₅.²⁰ Reaction of **5** with NaN₃ in aqueous acetone afforded the white crystalline diazide, **6**, which underwent Curtius rearrangement upon heating in toluene affording crude diisocyanate **7**. Treatment of **7** with *tert*-butyl alcohol, unlike the *trans*-isocyanate,² gave less than 5% of the desired dicarbamate **8** and produced diazabicyclohexanes **9** and **10** as major products.

Dicarbamate 8 was rapidly hydrolyzed under acidic conditions to diamine 11. Although carbamate 9 was easily converted to 10, various attempts (both hydrolytic and reductive) to transform 10 to *cis*-diamine 11 failed. However, reaction of diisocyanate 7 with benzyl alcohol

Cyclopropylbis(dioxopiperazines)

gave a readily separable mixture (1:1 ratio) of the dibenzyl carbamate 12 and the bicyclic carbamate 13. Hydrogenolysis of 12 over Pd/C in EtOH-AcOH generated 11 in high yield. Addition of *cis*-diamine 11 to an aqueous solution of bromoacetate,²¹ under basic conditions, gave, upon acidification, crude tetraacid 14, which was isolated as its tetramethyl ester 15 following treatment with 2,2dimethoxypropane containing concentrated HCl. Saponification of 15 in methanolic NaOH gave tetraacid 14 in fair yield. Whereas attempts to cyclize *cis*-tetraester 15 under conditions similar to those used in the preparation of *trans*-2 were unsuccessful,² treatment of 15 with NH₂CHO-NaH in DME^{22,23} generated the desired bis-(dioxopiperazine) 3 in modest yield.



Structure Confirmation. The 90-MHz proton resonance spectrum for cyclopropane ring protons in trans-2, cis-3, and their diamine dihydrochloride precursors confirmed the structural assignments. For trans-2 and its diamine dihydrochloride precursor the expected AA'XX' pattern appeared as two deceptively simple triplets²⁴ having apparent $J_{AX} = 6$ Hz for trans-2 and $J_{AX} = 7$ Hz for the trans-diamine dihydrochloride. For the respective cis isomers the proton resonance patterns were considerably more complex. The computer-simulated analysis for the \mbox{ABX}_2 spectrum attributable to the cyclopropane ring proton resonance signals for cis-3 showed δ 0.56 for Hig proton resonance signals for the 0 biowed 0 close 0 H_{X_2} with $J_{AB} = -5.7 \text{ Hz}$, $^{25} J_{AX_2} = 5.3 \text{ Hz}$, and $J_{BX_2} = 7.7 \text{ Hz}$. Similarly, for the *cis*-diamine dihydrochloride precursor 11 the resonance signals were δ 1.27 for H_A, 1.52 for H_B, and 2.96 for H_{X₂} with $J_{AB} = -8.65$ Hz, $J_{AX_2} = 5.82$ Hz, and $J_{BX_2} = 8.68$ Hz. In both *cis*-3 and *cis*-11 the proton signal for H_A cis to the amino substituents appears at higher field than the geminal, trans H_B resonance signal. Interestingly, the $\Delta\delta$ (0.71) for the H_A resonance signals of the two cis isomers (3 and 11) is equal to the $\Delta\delta$ (0.71) for the H_B resonance signals in these compounds. Therefore, the $\Delta \delta$ for H_A vs. H_B in both compounds are identical (0.25 Hz). Although solvent effects (cis-3 in Me_2SO-d_6 and 11 in D_2O) are expected to affect the chemical shifts for these proton resonances, the same relative downfield shift for both H_A and H_B in 11 when compared to *cis*-3 likely is a reflection of the greater deshielding effect of the protonated diamino groups.

Biological Results. The effects of intraperitoneal administration of ICRF-159 (1) or the *trans*- and *cis*-cyclopropyl analogues (2 and 3, respectively) on metastases of bronchogenic adenocarcinoma (designated LG1002)²⁶ in inbred male Syrian golden hamsters are shown in Table I. The dose chosen (15 mg/kg) was previously observed to inhibit liver metastasis of a hamster lymphoma.¹⁵ Thus,

Table I.Effect of ICRF-159 and StereoisomericAnalogues 2 and 3 on Metastasis of a HamsterLung Adenocarcinoma

treatment ^a	no. of animals	no. of metastases ^t
ICRF-159 (1)	10	174.9 ± 31.5^{c}
cis-3	10	171.0 ± 26.7^{d}
trans-2	10	264.8 ± 23.9^{e}
CMC control	9	205 ± 24.4
saline control	9	211.6 ± 43.9

^a Stock suspensions (100 mL) of drug (2.8 mg/mL) containing 3 drops of concentrated HCl and 5% carboxy-methylcellulose. Solutions were warmed to 37 °C in a water bath prior to injection. Control solutions having no drug were utilized in a similar fashion. ^b Mean ± standard error. ^c p < 0.005 vs. CMC, ns vs. saline. ^d p < 0.025 vs. saline. ^e p < 0.001 vs. CMC, p < 0.01 vs. saline.

for these initial investigations no attempt was made to determine the optimal drug concentration nor the route of administration. Under conditions described in the Experimental Section both ICRF-159 (1) and cis-3 significantly reduced the number of lung metastases when compared against the carboxymethylcellulose (CMC) vehicle control. Only *cis*-3 showed a significant reduction in metastases when compared against the saline control. However, *trans-2* administration produced a significantly greater number of lesions than those found in the lungs of animals from the other treatment groups. Additionally, trans-2 stimulated the growth of the primary tumor whereas ICRF-159 and cis-3 had no effect on primary tumor growth. The tumor growth in the latter animals was not different than that observed in animals receiving saline. Tumors appeared 3 to 4 days earlier in the *trans-2* treated animals and grew to a larger size $(>100 \text{ mm}^2)$ by the time of excision on the 28th day.

Discussion

To the best of our knowledge, these results are the first describing geometrical stereoselective control of metastases. Although Poggi et al.²⁷ have reported that (R)- and (S)-warfarin enantiomorphs show stereoselective antimetastatic properties, our studies have shown that one isomer (trans) stimulated and the other (cis) inhibited metastasis in the allogeneic hamster lung adenocarcinoma model. We are of the opinion that the results of this investigation are sufficiently encouraging to warrant further study of this family of compounds in several tumor systems with a view toward optimization of dose and route of administration and determination of the reasons why trans-2 and cis-3 show markedly different effects. Using Syrian hamster cells (V-79A) trans-2 was considerably less mutagenic and cytotoxic than ICRF-159.² One wonders whether the potentiating effects of *trans-2* may be related to an effect on cell volume and glycosaminoglycan biosynthesis as proposed by Lazo et al.¹⁸ for the effects of ICRF-159 on B16 melanoma cells, whereas cis-3 may selectively cause normalization of developing blood vessels in the primary tumor and thus inhibits metastases by mechanisms proposed by Hellmann and his collaborators.4,9

Experimental Section

Chemistry. Melting points were determined in open, glass capillaries on a Thomas-Hoover apparatus and are not corrected. Spectra were recorded on either a Perkin-Elmer 257 or Beckman Model 4230 spectrophotometer and Varian A-60 or Bruker HX-90E spectrometer. GLC utilized a Hewlett-Packard 402 biomedical gas chromatograph and elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn. Analyses were within $\pm 0.4\%$ of the calculated values. Mass spectra were

determined using a Du Pont Model 21-491 instrument by direct probe insertion (EI mode, 70 eV).

cis-1,2-Cyclopropanedicarboxylic acid (4) was prepared according to the method of Payne^{19a} and purified by chromatography on silica gel 60 (70–250 mesh), eluting with EtOActoluene-HCO₂H (49:33:1). An analytical sample was prepared by recrystallization from nitromethane, mp 139–140 °C (lit.^{19b} mp 139–142 °C).

cis-1,2-Cyclopropanedicarbonyl Dichloride (5). Diacid cis-4 (52.0 g, 0.40 mol) and PCl_5^{20} (249.6 g, 1.20 mol) were mixed and warmed on a steam bath for 5 h. After the mixture was cooled, excess PCl_5 was filtered and washed with Et₂O. Fractional distillation of the filtrate afforded 61.2 g (91.6%) of dichloride 5: bp 73–75 °C (1.5 mm); IR (neat) 1800 cm⁻¹ (C=O); NMR (CDCl₃) δ 1.5–2.2 (m, 2, CH₂), 2.67–3.07 (m, 2, CH).

cis-1,2-Cyclopropanedicarbonyl Diazide (6). Compound 5 (30.0 g, 0.18 mol) in acetone (60 mL) was added dropwise with stirring to a cooled (ice-salt bath) aqueous solution (100 mL) of NaN₃ (35.1 g, 0.54 mol). After complete addition, the reaction mixture was stirred at 0-5 °C for 2 h and then poured into ice-H₂O (400 mL). The aqueous phase was extracted with Et₂O (3 × 75 mL), and the combined organic layers were washed with cold H₂O (50 mL) and then dried (MgSO₄). Removal of volatiles under reduced pressure ($T \le 35$ °C) gave an oil which crystallized upon trituration with hexane (200 mL) to afford 28.2 g (87%) of diazide 6: mp 37-38 °C; IR (neat) 2140 (-N=N=N), 1720 cm⁻¹ (C=O).

cis-1,2-Cyclopropane Diisocyanate (7). Azide 6 (46.0 g, 0.25 mol) in Na-dried toluene (500 mL) was heated on a steam bath until N₂ evolution ceased (3 h). Removal of solvent under reduced pressure gave crude diisocyanate 7 (IR 2280 cm⁻¹ for NCO) which was used in subsequent reactions without further purification.

Reaction of cis-Diisocyanate 7 with tert-Butyl Alcohol. Crude diisocyanate 7 (obtained from 46 g of diazide 6) was refluxed in t-BuOH (500 mL) for 4 h, followed by removal of solvent under reduced pressure. The resulting residue was slurried in a small amount of ethyl acetate and filtered. Fractional crystallization of the precipitate from benzene gave 2-(2-methyl)propyl cis-3-oxo-2,4-diazabicyclo[3.1.0]hexane-2-carboxylate (9) [mp 123-124 °C; IR (KBr) 1770 cm⁻¹ (C=O); NMR (CDCl₃) δ 0.33-1.05 (m, 2, CH₂), 1.56 [s, 9, C(CH₃)₃], 2.92-3.20 (m, 1, CHN), 3.53-3.85 $[m, 1, CHNCO_2C(CH_3)_3], 7.35$ (br s, 1, HN). Anal. $(C_9H_{14}N_2O_3)$ C, H, N] and cis-2,4-diazabicyclo[3.1.0]hexan-3-one (10) [mp 204-205 °C (MeOH); IR (KBr) 1690 cm⁻¹ (C=O); NMR $(Me_2SO-d_6) \delta 0.1-0.65 \text{ (m, 2, CH}_2), 3.05 \text{ (q, 2, CHN)}, 6.93 \text{ (br s,})$ 2, NH). Anal. $(C_4H_6N_2O)$ C, H, N]. A single product could be obtained by refluxing the crude mixture in MeOH containing several drops of concentrated HCl for 2 h, followed by recrystallization from MeOH, generating 14.0 g (50%) of 10.

Chromatography of the ethyl acetate filtrate on silica gel 60 using EtOAc–MeOH (19:1) as eluant gave 3.5 g (<5%) of di-*tert*butyl *cis*-1,2-cyclopropanediylbis(carbamate) (8) which was recrystallized from benzene: mp 125–126 °C; IR (KBr) 3370 (NH), 1705 cm⁻¹ (C==O); NMR (CDCl₃) δ 0.4–1.4 (m, 2, CH₂), 1.46 [s, 18, C(CH₃)₃]. 2.5–2.8 (m, 2, CHN), 4.85 (br s, 2, HN). Anal. (C₁₃H₂₄N₂O₄) C, H, N.

cis-1,2-Diaminocyclopropane Dihydrochloride (11) from Dicarbamate 8. Compound 8 (2.2 g, 8.0 mmol) was refluxed in MeOH (30 mL) containing concentrated HCl (3 mL) for 1 h. After removal of volatiles under reduced pressure, the resulting residue was recrystallized from MeOH–Et₂O to give 1.03 g (89%) of 11: mp >220 °C dec; IR (KBr) 3000–2400 cm⁻¹ (br, ⁺NH₃); NMR (D₂O²⁸) δ 1.18–1.83 (m, 2, CH₂), 3.01 (q, 2, CHN). Anal. (C₃H₁₀N₂Cl₂) C, H, N.

cis-1,2-Diaminocyclopropane Dihydrochloride (11) from Dicarbamate 12. Compound 12 (11.3 g, 0.033 mol) was dissolved in absolute EtOH (130 mL) containing AcOH (12 mL) and hydrogenated (40 psi, room temperature) in the presence of 10% Pd/C (1.0 g) for 2 h. After removal of catalyst, the solution was poured into ethereal HCl, cooled, and stirred for 0.5 h. The resulting precipitate was collected and washed thoroughly with absolute EtOH-Et₂O (1:4) to give 4.5 g (93.7%) of 11 which was used without further purification in subsequent reactions. An analytical sample could be prepared as previously described.

Dibenzyl cis-1,2-Cyclopropanediylbis(carbamate) (12). Crude diisocyanate 7 (from 38.5 g, 0.213 mol, of diazide 6) was heated to 100 °C in benzyl alcohol (600 mL) for 2 h (IR absorption at 2280 cm⁻¹ was absent) and then allowed to come to room temperature overnight. After filtering, the resulting precipitate was slurried in MeOH and filtered. Recrystallization from CHCl₃-hexane gave 30.2 g (41.8%) of dicarbamate 12: mp 167–168 °C; IR (KBr) 3320 (NH), 1695 cm⁻¹ (C==O); NMR (CDCl₃) δ 0.5–1.5 (m, 2, CH₂), 2.55–2.85 (m, 2, CHN), 5.10 (s, 4, PhCH₂), 7.35 (s, 10, aromatic). Anal. (C₁₉H₂₀N₂O₄) C, H, N.

Benzyl cis-3-Oxo-2,4-Diazabicyclo[3.1.0]hexane-2carboxylate (13). Concentration of the filtrate and MeOH washings from the isolation of crude carbamate 12 afforded a residue which crystallized from CHCl₃-hexane to give 21 g (42.7%) of pure 13: mp 102-103 °C; IR (KBr) 1770 cm⁻¹ (C=O); NMR (CDCl₃) δ 0.4-1.0 (m, 2, CH₂), 2.9-3.23 (m, 1, CHN), 3.6-3.9 (m, 1, CHN), 5.30 (s, 2, PhCH₂), 7.38 (s, 5, aromatic). Anal. (C₁₂-H₁₂N₂O₃) C, H, N.

cis-2,2',2'',2'''-(1,2-Cyclopropanediyldinitrilo)tetrakis-(acetic acid) (14). Tetraester 15 (0.9 g, 2.5 mmol) was stirred overnight at 45–50 °C in a mixture of MeOH (20 mL) and 1 N aqueous NaOH (40 mL). After cooling, the pH was adjusted to 2–3 using dilute aqueous HCl. The volatiles were removed under reduced pressure and the solids were redissolved in H₂O (15 mL). The pH of this solution was adjusted to 1.5 with concentrated HCl, and the solution was kept at 4 °C for 3 days. The resulting crystals were collected and washed with cold H₂O (4:1) afforded transparent crystals: mp 166–168 °C dec; IR (KBr) 1750, 1700 cm⁻¹ (CO₂H); NMR (D₂O²⁸–NaOH, 90 MHz) δ 0.57–0.88 (m, 2, CH₂), 2.00 (q, 2, CHN), 3.62 (s, 8, CH₂). Anal. (C₁₁H₁₆N₂O₈) C, H, N.

Tetramethyl cis-2,2',2'',2'''-(1,2-Cyclopropanediyldinitrilo)tetrakis(acetate) (15). Bromoacetic acid (8.34 g, 0.06 $(mol)^{21}$ was dissolved in H₂O (16 mL), cooled in an ice bath, and neutralized (pH 7-8) by the dropwise addition of 6 N NaOH. To this solution (under N_2) was added 11 (1.45 g, 0.01 mol) in small portions. The solution was kept at pH 7-8 by concurrent dropwise addition of 6 N NaOH. After complete addition, the temperature was raised to 45-50 °C and the pH was adjusted and maintained at 10.5-11.5 by the addition of 6 N NaOH. After 2 h, the consumption of alkali decreased and only small amounts were required over the next 5 h. After stirring overnight at room temperature, the reaction mixture was cooled and acidified to pH 1.5 with concentrated HCl. Volatiles were removed under reduced pressure and the resulting residue was stirred overnight at room temperature in 2,2-dimethoxypropane (250 mL) containing concentrated HCl (10 mL). Upon removal of solvent under reduced pressure, the residue was dissolved in ice-H₂O and extracted with Et₂O (3×50 mL). The aqueous phase was made basic with Na₂CO₃ and extracted with Et₂O (3×50 mL). The organic layers were combined, dried (MgSO₄), and concentrated to afford 2.85 g (80%) of an oily product, 15, which was used without further purification. Distillation gave an analytical sample: bp 120-130 °C (0.05 mm); IR (neat) 1740 cm⁻¹ (ester); NMR (CDCl₃) δ 0.48–1.00 (m, 2, CH₂), 2.51 (q, 2, CHN), 3.68 (s, 20, CH_2 and CH_3). Anal. $(C_{15}H_{24}N_2O_8)$ C, H, N.

cis-4,4'-(1,2-Cyclopropanediyl)bis(2,6-piperazinedione) (3). To 57% NaH (1.48 g) dispersion in mineral oil in DME (50 mL, dried over LiAlH₄), heated to 95 °C under N₂, was added, dropwise with stirring, a mixture of 15 (2.78 g, 7.7 mmol) and formamide (1.4 g, 31 mmol) in DME (30 mL).^{22,23} After complete addition, the reaction mixture was refluxed for 2 h, followed by removal of volatiles under reduced pressure. The resulting paste was suspended in Et₂O and ice-H₂O (50 mL) was slowly added with cooling. The aqueous phase was filtered, acidified (pH 4) with aqueous HCl, and cooled (4 °C) overnight to give a crude product which was crystallized from Me₂SO-MeOH affording 0.75 g (36.5%) of 3: mp >230 °C; IR (KBr) 1730, 1690 cm⁻¹ (imide); NMR (Me₂SO-d₆, 90 MH₂) δ 0.46-0.97 (m, 2, CH₂), 1.94 (q, 2, CHN), 3.43 (s, 8, CH₂), 11.07 (s, 2, NH). Anal. (C₁₁H₁₄N₄O₄) C, H, N.

Comparative mass spectra of major ions $[m/e \ (\% \ ICRF-159, \% \ 2, \% \ 3)]$ for bis(dioxopiperazines) show 268 (M⁺) (0, 1.2, 3.4), 267 (0.5, 5.7, 10.2), 154 (5.8, 4.5, 6.3), 153 (71.5, 52.7, 63.2), 141 (68.1, 1.5, 2.3), 140 (6.5, 5.0, 6.0), 127 (100.0, 100.0, 100.0), 113 (5.3, 2.3, 3.0).

Biology. Inbred male Syrian golden hamsters, strain LSH/ LAK, were obtained from Charles River Laboratories (Lakeview,

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N.J.). Animals were housed five per cage under standard laboratory conditions and were used when 7-12 weeks of age.

The tumor cell line used in this study was a broncogenic adenocarcinoma, designated LG1002.²⁶ This line was induced in an outbred Syrian golden hamster by the intratracheal instillation of benzo[a]pyrene adsorbed to ferric oxide particles and suspended in saline.²⁹ The tumor was maintained by serial passage in the hamster cheek pouch. Tumor cell inocula were prepared by digesting small tumor pieces with trypsin and suspending the cells at the desired concentration in Hank's balanced salt solution (BSS).

Groups of hamsters averaging approximately 93 g each were injected intraperitoneally with 15 mg/kg of the compound being tested (see Table I, footnote a). Animals were treated every 48 h for 4 weeks and the last treatment occurred 48 h prior to excision of tumor. Growth of tumor cell inocula, given intradermally in the back, was monitored by measuring the greatest and the least diameters of the tumor nodule with calipers. Tumor size was expressed as the product of the two diameters.

Intradermal tumors were excised 4 weeks after implantation. The wound was inspected for the presence of subcutaneous tumor growth. The wound edges were apposed and fastened with sterile skin clips (Clay Adams, Parsippany, N.J.). Animals were checked daily for regrowth of the tumor at the excision site and sacrificed 7 days later. To enumerate the number of metastases, the lungs were cleared of blood by severing the dorsal aorta and injecting 3-5 mL of saline into the right ventricle. They were then removed, fixed as described by Williams and Nettesheim,³⁰ and stained and cleared according to the method of Yuhas.³¹ Mean tumor sizes and number of metastasis were compared using Student's t test.

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