Vol. 136, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1986 Pages 64-71

AURINTRICARBOXYLIC ACID AND EVANS BLUE REPRESENT TWO DIFFERENT CLASSES OF ANIONIC COMPOUNDS WHICH SELECTIVELY INHIBIT THE CYTOPATHOGENICITY OF HUMAN T-CELL LYMPHOTROPIC VIRUS TYPE III/LYMPHADENOPATHY-ASSOCIATED VIRUS

Jan Balzarini*,†, Hiroaki Mitsuya‡, Erik De Clercq† and Samuel Broder‡

†Rega Institute for Medical Research, University of Leuven,
B-3000 Leuven, Belgium

‡Clinical Oncology Program, National Cancer Institute,
Bethesda, Maryland 20205

Received February 20, 1986

SUMMARY Aurintricarboxylic acid, an anionic triphenylmethane dye, and Evans Blue, an anionic compound structurally related to suramin, are, like suramin itself, inhibitors of human T-cell lymphotropic virus type III (HTLV-III)/-lymphadenopathy-associated virus (LAV) in vitro. These compounds may be targeted, at least in part, at the HTLV-III/LAV reverse transcriptase. The lack of any appreciable cytostatic action of aurintricarboxylic acid, Evans Blue and suramin against several murine and human cell lines, their inability to inhibit cellular DNA, RNA and protein synthesis, and their high lethal dose-50 (⩾ 0.340 g/kg) for NMRI mice point to the selectivity of the compounds as inhibitors of HTLV-III/LAV.

Acquired immunodeficiency syndrome (AIDS) is a pandemic immunosuppressive disease which results in high susceptibility to unusual forms of certain neoplasms and life-threatening infections with opportunistic organisms.[1-3] The identification of a retrovirus, referred to as human T-cell lymphotropic virus type III (HTLV/III)/lymphadenopathy-associated virus (LAV) as the etiologic agent of this disease[3-6] has prompted the search for agents that may be useful in the prevention and/or therapy of AIDS.[7-14] Since continuous viral infection of T-cells seems necessary in the pathogenesis of AIDS, reverse transcriptase may be considered as a potential target for anti-HTLV-III/LAV activity. Indeed, most agents that have so far been proven effective in inhibiting the replication of HTLV-III/LAV, i.e. HPA-23,[8,9] phosphonoformic acid[13], suramin[11,12] and 3'-azido-2',3'-dideoxythymidine[14], affect the viral reverse transcriptase. Although an inhibitory effect on the reverse transcriptase probably accounts for the observed inhibitory activity against virus replication, other possible mechanisms of action cannot be excluded.

*Author to whom correspondence should be addressed at the Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium.

Abbreviations : ATA, aurintricarboxylic acid; HTLV-III/LAV, human T-cell lymphotropic virus type III/lymphadenopathy-associated virus; LD_{50}, 50 % lethal dose; i.p., intraperitoneal; MLS, median life span.

0006-291X/86 $1.50

In 1984, we demonstrated that suramin, a drug known to inhibit the reverse transcriptase of a number of retroviruses,[15] blocks the in vitro infectivity and cytopathic effect of HTLV-III/LAV.[11] Also, Broder et al.[16] found that suramin exerted an in vivo virustatic effect on HTLV-III/LAV in AIDS patients, although no significant clinical or immunological improvement was observed using a short-term treatment regimen (5 weeks). Recently, we compared suramin and a series of selected compounds for their inhibitory effects on HTLV-III/-LAV,[17] and found that Evans Blue and Direct Yellow-50, like suramin, inhibit HTLV-III/LAV-induced cytopathogenicity. We now report that the triphenylmethane dye, aurintricarboxylic acid (ATA) can also be considered as a potent and selective inhibitor of the in vitro cytopathogenicity and infectivity of HTLV-III/LAV. Its inhibitory effect against HTLV-III/LAV, its cytostatic and antimetabolic effect against several murine and human tumor cell lines, and its acute toxicity in NMRI mice were compared with those of suramin, Evans Blue and aurin, the latter being the decarboxylated derivative of ATA.

MATERIALS AND METHODS

Cells. The murine cell lines leukemia L1210 and mammary carcinoma FM3A and the human cell lines B-lymphoblast Raji, T-lymphoblast Molt/4F and Erythroleukemia K527 were grown in Eagle's minimum essential medium, supplemented with 10 % (v/v) inactivated calf serum (Gibco Bio-Cult, Glasgow, Scotland, U.K.), 2 mM L-glutamine (Flow Laboratories, Irvine, Scotland, U.K.), and 0.075 % (w/v) $NaHCO_3$ (Flow Laboratories). For the Raji cells, growth medium was supplemented with 1 % THM (5 mM TES, 7.5 mM HEPES and 5 mM MOPS) buffer (pH 5.3). Human ATH8 cells are an HTLV-I-transformed, clonal population of T-cells with high susceptibility to the cytopathic effect of HTLV-III. ATH8 cells were grown in RPMI-1640 medium (Flow Laboratories) containing 15 % (v/v) fetal calf serum, 15 % (v/v) tissue culture growth factor (TCGF) (Cellular Products), 4 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin.

Compounds. Suramin was obtained from Mobay Chemical Corporation, FBA Pharmaceuticals, or from Bayer AG through the courtesy of Dr. G. Streisle (Bayer AG, Wuppertal, West-Germany). Evans Blue, aurintricarboxylic acid and aurin were from Aldrich Chemical Company (Milwaukee, Wisc., USA). [Methyl-^{3}H]dThd (specific radioactivity 47 Ci/mmol), [5-^{3}H]Urd (specific radioactivity 25 Ci/mmol) and [4,5-^{3}H]leucine (specific radioactivity 120 mCi/mmol) were obtained from Amersham International Limited (Amersham, England).

Cytostatic and antimetabolic assays. The procedure for measuring inhibition of cell proliferation and for determining the incorporation of radiolabeled [methyl-^{3}H]dThd, [5-^{3}H]Urd and [4,5-^{3}H]leucine into TCA-insoluble material of murine leukemia L1210 cells has been described previously.[18,19]

Inhibition of the cytopathic effect of HTLV-III/LAV upon infection of ATH8 cells. The procedure for measuring the cytopathic effect of HTLV-III/LAV upon infection of ATH8 cells has been described previously.[11,12] Briefly, cells were suspended in growth medium and pretreated with polybrene at 2 µg/ml for 30 min at 37°C. After centrifugation and resuspension at 2 x 10^5 cells/culture tube, cells were pelleted and infected with 5 x 10^3 HTLV-III/LAV virions per cell for 60-90 min at 37°C. After infection, cells were resuspended in culture medium (at 2 ml/tube) in the presence or absence of the test compounds and incubated for several days at 37°C. Mock-infected cells were incubated in the presence of identical concentrations of the test compounds. At 7-10 days after virus infection, the number of viable cells was counted in both the virus-infected and mock-infected cell groups.

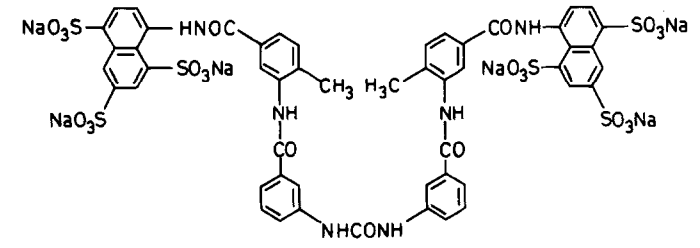
Inhibition of p24 expression in H9 cells infected with HTLV-III/LAV. The procedure for measuring p24 expression in H9 cells infected with HTLV-III/LAV has been described by Popovic et al.[20] Briefly, H9 cells were exposed for 2 hr at 37°C to suramin (10 µg/ml, 50 µg/ml), Evans Blue (10 µg/ml, 100 µg/ml), ATA (10 µg/ml, 20 µg/ml, 50 µg/ml) or aurin (5 µg/ml, 10 µg/ml, 20 µg/ml); then polybrene was added at 2 µg/ml for 30 min, the cells were pelleted, exposed to HTLV-III/LAV virions for 90 min, resuspended in fresh culture medium and further incubated in culture tubes at 37°C. Cells were continuously exposed to each concentration of the test compound. On day 9 the percentage of the target cells expressing p24 gag protein of HTLV-III/LAV was determined by indirect immunofluorescence microscopy by using anti-HTLV-III/LAV p24 murine monoclonal antibody (M26).[21]

In vivo toxicity tests. An equivalent number of 3 male and 3 female NMRI (Naval Medical Research Institute) mice weighing 10 to 12 g were used for the in vivo toxicity tests. In a first set of experiments, mice were given a single intraperitoneal (i.p.) dose of 1000, 500 or 250 mg/kg of suramin, Evans Blue or ATA. From the data obtained, the lethal dose-50 (LD_{50}) was calculated. In a second set of experiments, mice were injected i.p. until death with a daily dose of 500, 250 or 125 mg/kg of suramin; 250, 125, 62.5 or 31.2 mg/kg of ATA; and 125, 62.5 or 31.2 mg/kg of Evans Blue. From the data obtained, the Median Life Span (MLS) was determined.

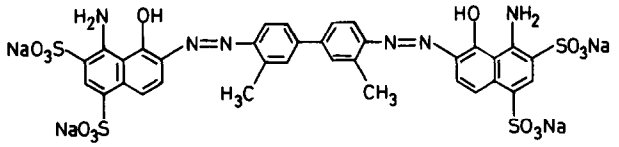
RESULTS

As is clear from Fig. 1, suramin and Evans Blue belong to a class of anionic compounds which are structurally distinct from those of the triphenylmethane derivatives ATA and aurin. When Evans Blue, suramin, ATA and aurin were evaluated for their inhibitory effects on the cytopathogenicity of HTLV-III/LAV for ATH8 cells, Evans Blue proved quite effective in protecting the cells against the cytopathic effects of the virus (Fig. 2). At 50 and 25 µg/ml, Evans Blue completely protected the cells and, even at 10 µg/ml, it effected 50 % protection. When assayed in parallel, suramin completely protected the cells at 50 µg/ml, but at 25 µg/ml it was only 34 % protective. Suramin completely suppressed the expression of p24 gag protein at 50 µg/ml;[17] at 10 µg/ml, it showed only a modest and temporary protective effect.[11] In contrast, Evans Blue was totally protective at a concentration of 10 µg/ml.[17]

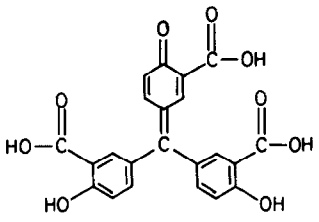
ATA showed marked protection against the cytopathogenicity of HTLV-III/LAV for ATH8 cells (Fig. 2). At a concentration of 20 µg ATA per ml, the cells were completely protected. At 10 µg/ml ATA effected 24 % protection. At 50 µg/ml, ATA showed a slight reduction in the number of mock-infected cells. Aurin, a structural analogue of ATA which lacks the three carboxylic acid groups (Fig. 1), proved extremely cytotoxic at 10 µg/ml and totally inactive against HTLV-III/LAV. When evaluated for its inhibitory effects on p24 gag protein expression, ATA was totally protective at 50 µg/ml and 20 µg/ml, and effected almost 97 % protection at 10 µg/ml (data not shown). In contrast, aurin was only 10 % or 26 % protective at a concentration of 10 µg/ml and 5 µg/ml, respectively. At a higher dose (20 µg/ml) the effect of aurin on p24 gag protein expression could not be measured because of the cytotoxicity of the compound.



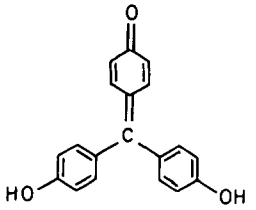
Suramin



Evans Blue



Aurintricarboxylic Acid



Aurin

Fig. 1. Structural formulae of suramin, Evans Blue, ATA and aurin.

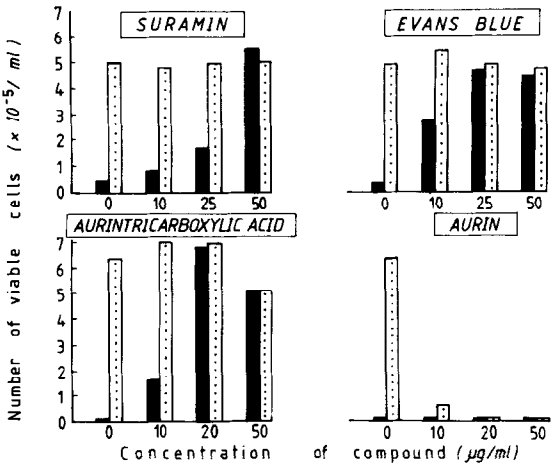


Fig. 2. Inhibition of the cytopathogenicity of HTLV-III/LAV for ATH8 cells by suramin, Evans Blue, ATA and aurin. Viability of the cells was measured after an incubation period of 7 days (suramin), 8 days (Evans Blue) or 10 days (ATA, aurin). Mock-infected cells, incubated in the presence of different concentrations of the test compounds (▣); HTLV-III/LAV-infected cells, incubated in the presence of different concentrations of the test compounds (■).

Table 1. Inhibitory effects of suramin, Evans Blue, ATA and aurin on tumor cell proliferation and metabolism

Compound	50 % Inhibitory dose (µg/ml) for cell growth				
	L1210	FM3A	RAJI	MOLT/4F	K 527
Suramin	670	>1000	150	716	> 1000
Evans Blue	407	383	180	316	453
ATA	620	507	230	752	712
Aurin	3.8	3.4	2.2	3.5	3.5

Compound	50 % Inhibitory dose (µg/ml) for incorporation into TCA-insoluble L1210 cell material		
	[Methyl-^{3}H]dThd into DNA	[5-^{3}H]Urd into RNA	[4,5-^{3}H]leucine into protein
Suramin	>1000	>1000	>1000
Evans Blue	>1000	>1000	>1000
ATA	>1000	>1000	862
Aurin	5.1	5.5	2.3

When examined for their inhibitory action on the proliferation of murine leukemia (L1210), murine mammary carcinoma (FM3A), human B-cell lymphoblast (Raji), human T-cell lymphoblast (Molt/4F) and human erythroleukemia (K 527) cells, aurin was considerably cytostatic (50 % inhibitory dose : about 3 µg/ml) (Table 1). Neither suramin, Evans Blue, or ATA demonstrated a significant cytostatic action. Nor did they prove inhibitory to DNA, RNA or protein synthesis (as monitored by the incorporation into L1210 cell TCA-insoluble material of [methyl-^{3}H]dThd, [5-^{3}H]Urd or [4,5-^{3}H]leucine, respectively). In contrast, aurin was inhibitory to DNA, RNA or protein synthesis at a concentration of about 2-5 µg/ml (Table 1).

The acute toxicity of suramin, Evans Blue and ATA was assessed in NMRI mice; the lethal dose-50 (LD_{50}) for a single intraperitoneal (i.p.) injection was 0.75, 0.34 and 0.34 g/kg, respectively. When injected i.p. daily at 500, 250, 125, 62 and 31 mg/kg until death, ATA was slightly more toxic than Evans Blue, while suramin was the best tolerated of the three compounds (Table 2). For example, with a daily dose regimen of 125 mg/kg, the median life span (MLS) of mice was 4.8, 5.5 and > 15 days for ATA, Evans Blue and suramin, respectively. No significant differences were noted in the MLS of male and female mice.

Table 2. Lethal effects of intraperitoneal administration of suramin, Evans Blue or ATA in NMRI mice

Compound	Dose (mg/kg/day)	Median life span (days)
ATA	250	3.5
	125	4.8
	62	9.1
	31	18.0
Evans Blue	125	5.5
	62	9.3
	31	> 20(*)
Suramin	500	4.3
	250	6.3
	125	> 20(*)

*Upon 15 daily administrations.

DISCUSSION

Evans Blue is structurally related to suramin but smaller in size, and contains only four sulfonic acid groups in stead of six (Fig. 1). The fact that Evans Blue is at least as effective as suramin in inhibiting the cytopathogenicity and p24 gag protein expression of HTLV-III/LAV clearly points to this class of anionic compounds as a source for other, and possibly more potent or selective, inhibitors of HTLV-III/LAV. Therefore, structure-activity relationship studies with this class of compounds seem highly warranted.

The triphenylmethane dye ATA is also a potent and selective inhibitor of the *in vitro* infectivity and replication of HTLV-III/LAV. This compound has previously been shown to block: (*i*) initiation of protein synthesis by preventing the attachment of mRNA to ribosomes;[22,23] (*ii*) RNA polymerase by interacting with the template binding site of this enzyme;[23,24] and (*iii*) RNA-directed DNA polymerase (reverse transcriptase)[23,24] by specifically reducing the affinity of the reverse transcriptase for the DNA primer molecule.[23] Based on these data, the most likely target for the inhibitory effect of ATA on HTLV-III/LAV replication is the viral reverse transcriptase. ATA may be considered as the prototype of a new class of anionic compounds, namely those derived from triphenylmethane (Fig. 1), that should be further pursued for their inhibitory effects on HTLV-III/LAV. Aurin, a structural analogue of ATA lacking the 3 carboxylic acid groups, was totally inactive against HTLV-III/-LAV. This compound has also proven inactive against Rauscher murine leukemia virus reverse transcriptase.[22] Thus, the anionic properties of this class of compounds appear to contribute to a large extent to their activity as HTLV-III/LAV inhibitors.

The failure of ATA and Evans Blue to inhibit the proliferation of murine leukemia, murine mammary carcinoma, human B-lymphoblast, human T-lymphoblast and human erythroleukemia cells, their inability to inhibit cellular macromolecule (DNA, RNA or protein) synthesis, their high acute LD_{50} when administered as a single i.p. dose to NMRI mice and, similarly, the relatively high daily doses that are required for lethality, all point to the selectivity of ATA and Evans Blue as inhibitors of HTLV-III/LAV.

In conclusion, ATA and Evans Blue inhibit the infectivity and replication of HTLV-III/LAV for T-cells at concentrations which are not toxic to the host cells. They belong to two different classes of anionic compounds, which both represent interesting leads in current attempts to develop an effective chemotherapy for retrovirus-associated diseases (i.e. AIDS) in humans.

ACKNOWLEDGMENTS

This research was supported by the Belgian F.G.W.O. (Fonds voor Geneeskundig Wetenschappelijk Onderzoek) (krediet no. 3.0040.83) and the Belgian G.O.A. (Geconcerteerde Onderzoeksacties) (Conventie no. 85/90-79). We thank Lizette Van Berckelaer for excellent technical help and Christiane Callebaut for fine editorial assistance.

REFERENCES

1. Wong-Staal, F., and Gallo, R.C. (1985) Nature 317, 395-403.
2. Curran, J.W., Morgan, W.M., Hardy, A.M., Jaffe, H.W., Darrow, W.W., and Dowdle, W.R. (1985) Science 229, 1352-1357.
3. Broder, S., and Gallo, R.C. (1985) Ann. Rev. Immunol. 3, 321-336.
4. Barré-Sinoussi, F., Chermann, J.C., Rey, R., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W., and Montagnier, L. (1983) Science 220, 868-871.
5. Gallo, R.C., Sarin, P.S., Gelmann, E.P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V.S., Mann, D.L., Sidhu, G., Stahl, R.E., Zolla-Pazner, S., Leibowitch, J., and Popovic, M. (1983) Science 220, 865-867.
6. Broder, S., and Gallo, R.C. (1984) New Engl. J. Med. 311, 1292-1297.
7. McCormick, J.B., Mitchell, S.W., Getchell, J.P., and Hicks, D.R. (1984) Lancet ii, 1367-1369.
8. Dormont, D., Spire, B., Barré-Sinoussi, F.C., Montagnier, L., and Chermann, J.C. (1985) Ann. Microbiol. 136E, 75-84.
9. Rozenbaum, W., Dormont, D., Spire, B., Vilmer, E., Gentilini, M., Griscelli, C., Montagnier, L., Barré-Sinoussi, F., and Chermann, J.C. (1985) Lancet i, 450-451.
10. Ho, D.D., Rota, T.R., Kaplan, J.C., Hartshorn, K.L., Andrews, C.A., Schooley, R.T., and Hirsch, M.S. (1985) Lancet i, 602-604.
11. Mitsuya, H., Popovic, M., Yarchoan, R., Matsushita, S., Gallo, R.C., and Broder, S. (1984) Science 226, 172-174.
12. Mitsuya, H., Matsushita, S., Harper, M.E., and Broder, S. (1985) Cancer Res. 45 (suppl.), 4583s-4587s.
13. Sandstrom, E.G., Kaplan, J.C., Byington, R.E., and Hirsch, M.S. (1985) Lancet i, 1480-1482.
14. Mitsuya, H., Weinhold, K.J., Furman, P.A., St. Clair, M.H., Nusinoff Lehrman, S., Gallo, R.C., Bolognesi, D., Barry, D.W., and Broder, S. (1985) Proc. Nat. Acad. Sci. USA 82, 7096-7100.
15. De Clercq, E. (1979) Cancer Letters 8, 9-22.

16. Broder, S., Yarchoan, R., Collins, J.M., Lane, H.C., Markham, P.D., Klecker, R.W., Redfield, R.R., Mitsuya, H., Hoth, D.F., Gelmann, E., Groopman, J.E., Resnick, L., Gallo, R.C., Myers, C.E., and Fauci, A.S. (1985) Lancet ii, 627-630.
17. Balzarini, J., Mitsuya, H., De Clercq, E., and Broder, S. (1986) Int. J. Cancer, in press.
18. De Clercq, E., Balzarini, J., Torrence, P.F., Mertes, M.P., Schmidt, C.L., Shugar, D., Barr, P.J., Jones, A.S., Verhelst, G., and Walker, R.T. (1981) Mol. Pharmacol. 19, 321-330.
19. Balzarini, J., De Clercq, E., Ayusawa, D., and Seno, T. (1984) FEBS Letters 173, 227-232.
20. Popovic, M., Sarngadharan, M.G., Read, E., and Gallo, R.C. (1984) Science 224, 497-500.
21. Veronese, F.D., Sarngadharan, M.G., Rahman, R., Markham, P.D., Popovic, M., Bodner, A.J., and Gallo, R.C. (1985) Proc. Natl. Acad. Sci. USA 82, 5199-5202.
22. Grollman, A.P., and Stewart, M.L. (1968) Proc. Natl. Acad. Sci. U.S.A. 61, 719-725.
23. Givens, J.F., and Manly, K.F. (1976) Nucleic Acids Res. 3, 405-418.
24. Liao, L.L., Horwitz, S.B., Huang, M.T., Grollman, A.P., Steward, D., and Martin, M.J. (1975) J. Med. Chem. 18, 117-120.