Eur J Cancer, Vol. 26, No. 6, pp. 709–714, 1990.
Printed in Great Britain

0277-5379/90$3.00 + 0.00
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Genetic Toxicology of Tricyclic Carboxamides, a New Class of DNA Binding Antitumour Agent

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(*N*-[2-(Dimethylamino)ethyl]acridine-4-carboxamide (acridine carboxamide; NSC 601316) is an acridine-derived experimental antitumour agent with curative properties against Lewis lung carcinoma in mice. Although it intercalates into DNA and also appears to interact with topoisomerase II, its DNA binding properties appear distinct from other acridine derivatives such as the clinical antitumour drug, amsacrine. The mutagenic properties of acridine carboxamide, together with three related compounds containing either 9-aminoacridine or phenazine chromophores, were studied at the 6-thioguanine and ouabain loci in cultured V79 Chinese hamster fibroblasts. Each compound, when tested at concentrations causing up to 90% kill, had weak but significant activity at the 6-thioguanine but not at the ouabain locus. All drugs were potent inducers of micronuclei, indicating high clastogenic activity. There was a highly significant relationship between mutation frequency (as resistance to 6-thioguanine) and either cytotoxicity (measured as D_{37} in a clastogenicity assay) or clastogenicity. A broader range of compounds was also tested for microbial mutagenicity. In *Salmonella typhimurium* strains, none were mutagenic in TA98, TA100 or TA102 but several were mutagenic in TA1537, a frameshift tester strain. Some drugs also caused 'petite' mutagenesis in *Saccharomyces cerevisiae*. In general, compounds with the phenazine chromophore, which has no positive charge, were the most mutagenic in these systems. However, activity was not related to mammalian mutagenicity or antitumour effect. The results suggest that in mammalian cells, the cytotoxicity, clastogenicity and mutagenic activity of these drugs are mediated by similar mechanisms to those for amsacrine analogues, probably involving the enzyme DNA topoisomerase II.
Eur J Cancer, Vol. 26, No. 6, pp. 709—714, 1990.

INTRODUCTION

DNA intercalating agents comprise an important class of clinical antitumour agents, and rely in general on the nuclear enzyme DNA topoisomerase II for their activity [1]. A programme in this laboratory to prepare DNA binding compounds active against experimental solid tumours led to the acridine derivatives amsacrine [2], now used clinically for treatment of leukaemia [3], and CI-921, currently under clinical trial [4]. A search for related compounds with antitumour activity led to the synthesis of a series of derivatives of *N*-[2-(dimethylamino)-ethyl]-9-aminoacridine-4-carboxamide [5, 6]. Consideration of structure–activity relationships in this series led to *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide (acridine carboxamide; NSC 601316; see Fig. 1 for structures), which is curative against Lewis lung tumours in mice [7]. Other compounds in which the acridine chromophore has been replaced by a phenazine group and other tricyclic moieties have now been developed and found also to have high antitumour activity [8, 9].

Acridine carboxamide intercalates into double-stranded DNA with an association constant similar to that of CI-921 [5]. It targets the enzyme DNA topoisomerase II but differs from many other drugs acting on this enzyme by having no easily oxidizable or reducible groups [10]. In addition, acridine carboxamide is active against an actinomycin D-resistant P388 leukaemia line which is cross-resistant to vincristine, etoposide and doxorubicin [10], suggesting that it may be useful against

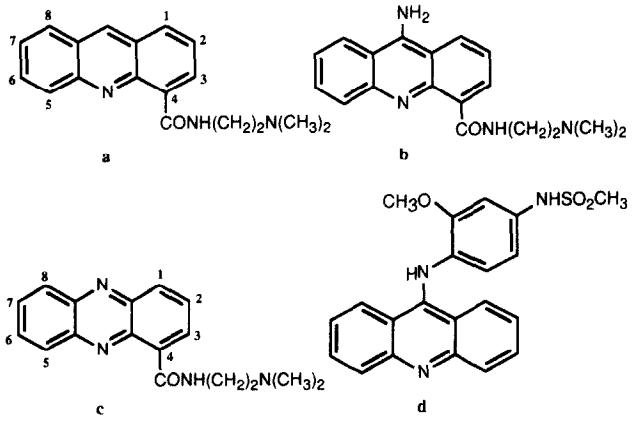

Fig. 1. Structures of tricyclic carboxamides where a = acridine carboxamide, b = aminoacridine carboxamide, and c = phenazine carboxamide. The related compound, amsacrine (d), is also shown.

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multidrug-resistant cells. These together with other properties [7, 11] have led to the consideration of acridine carboxamide as a candidate drug for clinical trial.

We have now investigated the genetic toxicology of some of these compounds in order to determine whether mutagenicity, which is a drawback clinically because of potential carcinogenicity [12] and induction of resistance to other clinical agents [13], can be separated from antitumour effects. The mutagenic properties of acridine carboxamide and related compounds (see Fig. 1 for structures), have also been compared with those previously reported for amsacrine and its analogues [14–16]. Drugs have been tested for mutagenesis in three strains of *Salmonella typhimurium* and for the induction of respiratory deficient mutants of the yeast *Saccharomyces cerevisiae*. The mammalian mutagenicity of four drugs has been quantitated by measuring the induction of 6-thioguanine and ouabain resistance in V79 Chinese hamster cells. The clastogenic potential of the drugs has been estimated in the same experiments by scoring micronuclei in interphase cells.

MATERIALS AND METHODS

Chemicals

Tricyclic carboxamides were synthesized in this laboratory by Drs W.A. Denny, G.J. Atwell, B.D. Palmer and G.W. Rewcastle [5–8] and were pure as judged by thin-layer chromatography. 9-Aminoacridine, 4-nitro-*o*-phenylene diamine, sodium azide and ouabain were from Sigma, U.S.A. and 6-thioguanine was from Aldrich, U.S.A. Sterile stock solutions of 6-thioguanine (1 mg/ml in 0.5% Na_2CO_3) were stored at −20°C. Fresh solutions of all other chemicals were prepared immediately before use.

Microbial strains

S. typhimurium strains TA1537, TA98 and TA100 were kindly provided by Professor B.N. Ames, Department of Biochemistry, University of California, Berkeley, California, U.S.A. *S. cerevisiae* strain D5 [17] was kindly provided by Dr B.S. Cox, Botany School, Oxford University, U.K. Strains were stored as frozen stationary phase cultures in 1 ml aliquots (in 10% dimethylsulphoxide) at −80°C.

Microbial mutagenicity assays

For *S. typhimurium* assays, a vial was removed from −80°C storage, inoculated into fresh bacterial complete medium (20 ml) and grown until the optical density of culture gave an absorbance reading of between 0.11 and 0.12 at 654 nm. The well test, previously described [18], is a variation of the spot test as described by Maron and Ames [19] where a well cut in agar (overlaid with bacteria) is filled with the compounds being tested. The plate incorporation assay [19] was carried out as previously described [16] with 5×10^7 bacteria per plate. Each point was determined in triplicate on at least two occasions. Mutation frequencies were calculated from the slopes of linear regression lines in the dose range where the compound showed

Table 1. *Summary of published antitumour data for tricyclic carboxamides*

		L1210†	P388‡		Lewis lung‡	
Class	Substituents*	IC_{50} (μml/l)	OD (μmol/kg)	ILS (%)	OD (μmol/kg)	ILS (%)
a	—	100	180	87	270	—(6)§
	2-OCH_3	30	250	99	250	105(2)
	5-CH_3	4.3	79	87	120	183(4)
	5-OCH_3	12	76	88	110	54
b	—	15	12	98	12	NA
	5-CH_3	0.47	6.6	107	4.6	NA
	5-OCH_3	4.3	9.5	81	9.5	NA
	5-SO_2CH_3	2.8	65	138(5)	140	106(1)
	6-OCH_3	150	220	NA	220	NA
	7-OCH_3	670	32	NA	—	—
c	—	1700	450	88	450	57
	5-CH_3	42	190	45	190	62(1)
	5-OCH_3	48	280	106	280	128(4)
	5-OCH_2CH_3	61	270	92	400	84(4)
	7-OCH_3	4100	280	NA	420	NA
d	Amsacrine	12.5	17	78	17	42
e	9-Aminoacridine	1900	87	3		

*Based on Fig. 1.

†*In vitro* concentration for 50% growth inhibition with L1210 cells and 3 day exposure.

‡Activity against intraperitoneal P388 leukaemia and intravenous Lewis lung carcinoma [5–9]; drug dosage days 1, 5, 9 after tumor inoculation for P388 and days 5, 9, 13 for Lewis lung. OD = optimal dose; ILS = increase in lifespan over untreated mice; NA = not significantly active.

§Average numbers of long-term survivors in groups of six mice. NA = not significantly active.

little or no toxicity as judged by examination of the background bacterial lawn. 9-Aminoacridine was used as positive control for TA1537, 4-nitro-*o*-phenylene diamine for TA98 and sodium azide for TA100.

S. cerevisiae cells were used in a microtitre assay as previously described [20]. After growth for 2 h in the presence of various drug concentrations, cells were washed by dilution and plated on each of three YC plates [21]. These were incubated for 3 days at 30°C before being scored for survival and for 'petite' mutants.

Mammalian cells

The Chinese hamster fibroblast line V79-171b was originally obtained from Dr W.R. Inch, London, Ontario, Canada and was provided by Dr W.R. Wilson, Department of Pathology, University of Auckland Medical School. The growth medium was α-MEM without nucleosides or antibiotics, containing 10% v/v heat-inactivated foetal calf serum (Gibco, New Zealand). Cells were subcultured by trypsinization and dilution to 10^4 cells per 25 cm flask twice weekly. Cell cultures were maintained in humidified incubators with an atmosphere of 5% CO_2 in air at 37°C.

Mammalian mutagenesis assay

Exponential phase cultures initiated 24 h previously at 1.5×10^5 cells/ml in growth medium (α-MEM containing 10% foetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin; 10 ml per 100 mm Petri dish) were treated by addition of drug in prewarmed growth medium as previously described [14, 18]. Cell survival was assessed by plating up to 10^4 cells in plating medium (as above but containing 3% foetal calf serum; 5 ml per 60 mm Petri dish) and counting colonies 8 days later after staining with 0.5% methylene blue in 50% ethanol. The plating efficiency of the controls was in the range 60–90%. After drug treatment, cells were subcultured in growth medium to give approximately 10^6 clonogenic cells but not more than 4×10^6 cells in total per 100 mm Petri dish. Cells were subsequently maintained in exponential phase growth by subculturing to 10^6 cells every 2 days to allow expression of the mutant phenotype. After expression periods of 6 or 8 days, mutant cells were selected by plating 6×10^5 cells in 100 mm Petri dishes (four replicates per culture) containing 15 ml of plating medium with ouabain (3 mmol/l) or 6-thioguanine (5 μg/ml). At the same time, 100 mm dishes containing 15 ml plating medium were seeded with 120 cells (in triplicate for each culture) to determine the plating frequency at the time of mutant selection. Dishes were incubated for 11 days (selection dishes) or 8 days (non-selection dishes) to allow colony formation. The mutation frequency was calculated as the ratio of the plating efficiency in selective medium to that in unselected conditions. Standard errors for mutation frequency are based on colony counts in replicate selective and non-selective plates. The statistical significance of trends in mutation frequency with drug concentration was tested by linear regression analysis [14]. Agents were classed as mutagenic if the gradient of the regression line was significantly positive ($P \leq 0.05$).

Micronucleus assay

Micronuclei were studied in parallel with the mutagenesis experiments in order to correlate a measure of clastogenesis with

Table 2. Microbial mutagenicity and physicochemical properties of tricyclic carboxamides

Structure*	Substituent	*S. typhimurium** Max.	Rev/nmol	*S. cerevisiae*† D_{37}	P	Physicochemistry‡ R_m	log K	pK_a
a	—	2.6	0.4	1400	2.2	−0.2	6.12	3.54
	2-OCH_3	8	0.4	560	2.2	−0.3	6.80	3.34
	5-CH_3	13	9.7	120	14	−0.17	6.95	—
	5-OCH_3	14	5.9	570	76	−0.31	6.86	3.87
b	—	42	1.1	180	1.8	−1.11	7.08	8.30
	5-CH_3	>200	9.7	120	1.5	−1.02	7.55	8.01
	5-OCH_3	8.2	0.2	43	1.4	−1.06	7.62	7.80
	5-SO_2CH_3	14	0.2	920	0.9	−1.35	7.28	5.15
	6-OCH_3	14	0.2	57	1.1	—	—	—
	7-OCH_3	4.6	0.2	59	0.8	−0.90	7.11	7.74
c	—	170	11	1400	15	−0.29	6.58	—
	5-CH_3	32	22	1300	38	−0.23	6.76	—
	5-OCH_3	130	35	1300	64	−0.42	6.59	—
	5-OCH_2CH_3	>200	17	360	71	—	—	—
	7-OCH_3	170	8.4	2300	1.4	−0.27	6.40	—
d	Amsacrine	15	0.4	620	0	0.18	5.57	7.43
e	9-Aminoacridine	120	8.1	3100	0	−0.08	6.28	9.90

*Max. = maximum revertants per 10^6 bacteria plated; Rev/nmol = slope of regression line of revertants per 5×10^7 bacteria plated versus nmol of added drug [16].

†D_{37} = concentration (μmol/l) reduces *S. cerevisiae* survival to 37%; P = maximal induction (%) of 'petite' colonies [16].

‡R_m = relative chromatographic mobility; log K = logarithmic association constant for poly[dA-dT] determined at 0.01 ionic strength; pK_a = acridine base strength [5–9].

survival and mutation. Giemsa-stained cells were prepared 2 days after drug treatment for 60 min, as described previously [14]. Either 100 cells with micronuclei or 2000 cells in total were scored for each datum point. Estimates of the molar potency of clastogenesis were derived from the gradients of the linear regression lines giving best fit to the micronucleus induction frequency as a function of drug concentration. Drug concentrations giving survival levels of less than 20% were excluded from analysis, since there was clear evidence for non-linearity at high doses resulting from inhibition of progression of damaged cells through mitosis [14].

RESULTS

Relationship between antitumour activity and microbial mutagenicity

A range of compounds was selected which varied from inactive to curative against the Lewis lung carcinoma (Table 1) and which spanned a range of physicochemical properties (Table 2). Molar dose for active compounds varied over a 70-fold range and was correlated ($r = 0.64$; $P < 0.01$) with growth inhibitory concentrations (IC_{50} values) for cultured L1210 cells. None of the compounds, when assayed using well test methodology, caused demonstrable base-pair substitution mutagenesis in *S. typhimurium* TA100 or frameshift mutagenesis in TA98, although many of them were frameshift mutagens in TA1537 (data not shown). Quantitative data were then obtained for TA1537, shown in Table 2, show the phenazine series to be the most mutagenic, and the 5-methyl group to increase mutagenicity in the other two series. In contrast to data for amsacrine analogues [15, 16], there was no significant relationship between mutagenicity and either lipophilicity, DNA binding affinity, acridine base strength, dose potency or antitumour activity.

'Petite' mutagenesis in *S. cerevisiae* was also measured and is shown in Table 2. The phenazine series again showed the higher levels of mutagenicity than the other two series. In contrast to the amsacrine analogues [15, 16], no inverse relationship was found between 'petite' mutagenicity and bacterial frameshift mutagenicity.

Mutagenesis and micronucleus induction in V79 Chinese hamster cells

Cytotoxicity, clastogenicity and mutagenesis data in the V79 assays are summarized for a limited subset of these compounds together with amsacrine and 9-aminoacridine in Table 3. Cytotoxicity, as measured by D_{37} values, varied from 0.19 to 57 μmol/l. None of the drugs caused significant ouabain resistance in V79 cells, but all showed moderate ability to induce 6-thioguanine

*Table 3. Relationship between cytotoxic, clastogenic and mutagenic activity in V79 cells following 60 min drug treatment with tricyclic carboxamides**

			Clastogenesis†		Mutagenesis‡		
Drug†	Experiment	D_{37}* (μmol/l)	Molar potency	Activity at D_{37}	Molar potency	Activity at D_{37}	Mutants per 10^3 MN
a	1	1.8	1.3	2.7	22	20	0.74
	2	2.5	1.2	2.8	12	20	0.69
					12	17	0.59
b	1	0.35	9.5	5.9	44	20	0.34
	2	0.30	9.6	4.3	47	17	0.41
					54	13	0.30
b, 5-SO_2CH_3	1	0.78	3.9	2.9	35	23	0.81
	2	0.71	3.4	4.4	17	30	0.67
					25	35	0.79
c, 5-OCH_3	1	3.8	0.59	2.5	11	35	1.4
	2	3.3	0.59	1.6	4.7	32	2.0
					6.6	27	1.7
d	1	0.19	26	4.4	150	31	0.56
	2	0.27	ND	ND	130	37	ND
	3	0.27	26	7.0	65	16	0.25
e	1	57	0.029	1.6	NS	−0.2	—
	2	56	0.026	1.5	NS	−1.5	—

*Data for amsacrine (compound d) and 9-aminoacridine (compound e) are from Wilson *et al.* [14].
†Structures in Fig. 1.
†Molar potency gradient of linear regression percentage of cells with micronuclei (MN) to drug concentration (μmol/l), fitted to values of MN frequency below 9%; correlations were significant ($p < 0.05$) in all cases; activity at D_{37} = interpolated percentage of cells with MN (after subtraction of control frequency) at D_{37}.
‡Molar potency gradient of linear regression curve for mutants per 10^6 clonogenic cells versus drug concentration (μmol/l). NS = not significant; activity at D_{37} = interpolated mutants per 10^6 clonogenic cells (after subtraction of control frequency) at D_{37}, mutants per 10^6 MN = frequency of drug-induced mutations per 1000 induced MN.
§Expression time of 8 days; remainder have expression time of 6 days.

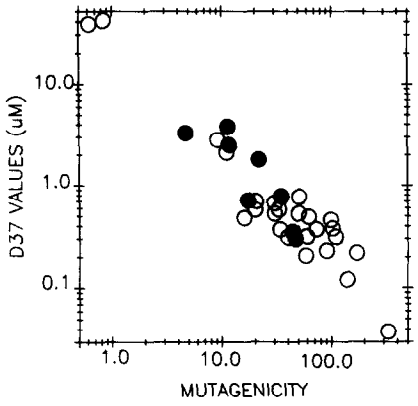

Fig. 2. Relationship between molar potency of mammalian cell mutagenicity (expression time 6 days) and cytotoxicity (as measured by D_{37} values) for the tricyclic carboxamide compounds in Table 3 (●). For comparison, previously reported data (○) for 9-anilinoacridine derivatives [14–16] are also presented.

resistance. Values for all drugs approximated 10–20-fold that of the negative control at the dose killing cells to 37% of the original level (Table 3). All drugs caused an increase in micronuclei to levels approximately 10–20-fold those of the negative controls although necessary doses for this activity varied widely between the drugs. The relationships between cytotoxicity, mutagenicity and clastogenicity are shown, together with corresponding data for amsacrine analogues [15, 16], in Figs 2 and 3.

DISCUSSION

The compounds chosen for this study included a number with high activity against the Lewis lung carcinoma in mice (Table 1), one (acridine carboxamide, Fig. 1) being capable of curing virtually 100% of animals when treatment is initiated on day 5 after inoculation of tumour cells [7, 11]. While compounds which are active against the Lewis lung tumour are also active against P388 leukaemia, the reverse is not true (Table 1), and a low degree of ionization of the chromophore (i.e. low pK_a value, see Table 2) appears to be one of the requirements for solid tumour activity [7, 8]. The phenazine carboxamide series (c), members of which do not have an ionizable chromophore, appear to have the highest overall microbial mutagenicity in both bacterial and yeast assays. This is probably not related to increased cellular uptake since the effective microbial toxicity of the phenazine derivatives falls into the same range as that for the other series.

In contrast to the results with microbial tests, the mammalian cell system shows a high degree of correlation between mutagenicity and cytotoxicity (Table 3, Figs 2 and 3). For the four tricyclic compounds examined, the logarithmic D_{37} value is correlated both with the logarithmic potency of clastogenesis ($r = -0.99$) and the logarithmic potency of micronucleus formation ($r = -0.87$). Furthermore, all values for the tricylic carboxamides lie on the same regression lines as were obtained for 9-anilinoacridine derivatives (Figs 4 and 5). Thus, as *in vitro* potency increases, mutagenic potential increases in parallel [16]. Mutagenicity at the HGPRT locus correlates very well with micronucleus induction in cultured cells. Although the ability of these compounds to transform cells in culture has not been measured, the close correlation found with anilinoacridine compounds between transformation potential and mutagenicity at the HGPRT locus argues strongly that mutagenicity, carcinogenicity (as measured by transformation) and cytotoxicity are closely related and likely to be mediated by the same mechanism.

In conclusion, it is likely that whereas microbial mutagenicity in two systems can be separated from mammalian mutagenicity, mammalian cytotoxicity and mutagenicity are closely linked. Since acridine carboxamide, like amsacrine and its analogues, acts on topoisomerase II as the target in cells, both mutagenicity and cytotoxicity may be linked to the formation of a 'cleavable complex' between DNA and topoisomerase II [22, 23] and subsequent irreversible DNA damage.

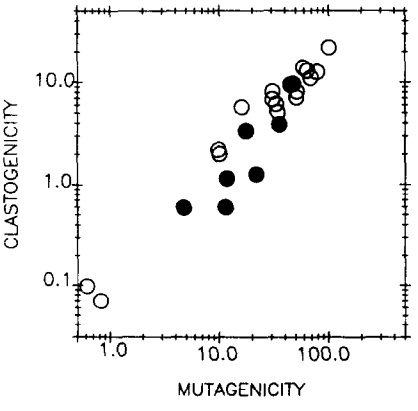

Fig. 3. Relationship between molar potency of mammalian cell mutagenicity (expression time 6 days) and clastogenicity (i.e. molar potency of micronucleus induction) for the tricyclic carboxamide compounds in Table 3 (●). For comparison, previously reported data (○) for 9-anilinoacridine derivatives [14–16] are also presented.

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Acknowledgements—Supported by the Cancer Society of New Zealand, its Auckland Division, and the Medical Research Council of New Zealand. The authors are grateful to Pamela Turner and Susan O'Rourke for help with the microbial assays, and to Lynden Hull for secretarial help.

Eur J Cancer, Vol. 26, No. 6, pp. 714–718, 1990.
Printed in Great Britain

0277–5379/90$3.00 + 0.00
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Cigarette Smoking and Bladder Cancer

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The relation between cigarette smoking and risk of bladder cancer was analysed in a case-control study in Northern Italy of 337 cases of histologically confirmed invasive bladder cancer and 392 controls admitted to the same network of hospitals with acute, non-neoplastic, non-urological conditions. Compared with never-smokers, the multivariate relative risk (RR) was 1.9 (95% confidence interval, CI 1.2–3.1) for ex-smokers and 3.3 (95% CI 2.2–5.0) for current smokers. The risk was directly and significantly related to duration of smoking (RR 3.5 for 30 years or more) and dose (RR 3.9 for 20 cigarettes per day or more), and consistent among strata of sex and age (though the RRs were systematically higher at older ages). Smokers of black tobacco only had a RR of 3.7, compared with 2.6 for smokers of blond cigarettes or mixed types . The interaction between tobacco and several occupations associated with bladder cancer risk fitted an additive rather than a multiplicative model: compared with non-exposed never-smokers, RR was 2.5 for exposed non-smokers, 2.8 for non-exposed smokers and 3.7 for occupationally exposed smokers.
Eur J Cancer, Vol. 26, No. 6, pp. 714—718, 1990.

INTRODUCTION

BLADDER CANCER is a known tobacco-related neoplasm, but the strength of the association is uncertain: the relative risks (RR) for smokers compared with non-smokers ranged between 1.4 and 2.9 in eight cohort studies, and the range of variation was even larger (1.2–7.3) in twenty case-control studies [1–12]. An Italian case-control study found a strong association between cigarette smoking and bladder cancer risk, with RR of 5.1 for smokers and of over 10 for heavy smokers (30 or more cigarettes per day) [3]. The proposed explanation for these elevated risks in an Italian population was the high frequency of dark tobacco smoked in the past in Italy [3, 4]. We present data from another