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Amino acid substitutions causing inhibitor resistance in TEM β-lactamases compromise the extended-spectrum phenotype in SHV extended-spectrum β-lactamases

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Three amino acid substitutions, Met-69→Ile, Arg-244→Ser and/or Asn-276→Asp, mediate inhibitor resistance (IR) in TEM β-lactamases. They were introduced in all possible combinations at homologous positions into either SHV-1 or the respective extended-spectrum β-lactamases (ESBLs), SHV-2 or SHV-5. Susceptibility testing of the resulting set of seven variants of each parental strain, all in an isogenic background, was performed. The phenotypes of the constructions revealed that most substitutions resulted in reduced resistance to most tested single β-lactam formulations. This decrease over-compensated for the expected increase in inhibitor resistance, so that most mutants showed no rise in resistance to inhibitor/β-lactam combinations, although increases of MICs from one- to 43-fold compared with the respective parental strains were also measured. Combination of several IR-determining substitutions impaired both phenotypes in the carrier strains even more. None of the 14 mutants derived from the ESBLs, SHV-2 and SHV-5, showed a clinically relevant combined ESBL–IR phenotype. These findings indicate that the SHV β-lactamase does not benefit proportionally from simultaneous substitution of residues relevant for the ESBL and the IR phenotype.

Introduction

The production of β-lactamases is the most frequently encountered mechanism of bacterial resistance to β-lactam antibiotics. Over 200 β-lactamases have been classified in four main groups and eight subgroups according to functional and structural characteristics.[1] Some variants, able to inactivate newer cephalosporins, are called extended-spectrum β-lactamases (ESBLs) and are predominantly plasmid mediated, and of growing clinical concern.[2,3] The majority of the ESBLs are derived through single amino acid substitutions in three non-ESBL parental enzymes, TEM-1, TEM-2 and SHV-1.[4,5] Since TEM- and SHV-ESBLs had been uniformly susceptible to β-lactamase inhibitors (e.g. clavulanic acid), inhibitor/β-lactam combinations were advocated as potential therapeutic alternatives, although with caution.[6,7]

During the 1980s, β-lactamase inhibitor-resistant (IR) strains began to emerge at low to moderate frequency. In most cases, they were found to overproduce an intrinsically inhibitor-susceptible β-lactamase (e.g. TEM-1), in sufficient quantity to overwhelm the inhibitor, leaving excess enzyme capable of destroying the accompanying β-lactam.[8,9] Since 1992, when TEM-30, the first mechanism-based IR variant emerged by a single amino acid substitution from TEM-1,[10] a whole new subgroup, designated 2br,[1] evolved. By 1999, this subgroup contained 18 variants derived from TEM-1 and one, TEM-50, derived from TEM-15, as well as one member of the SHV family, SHV-10.[11] The 18 TEM variants contain one or two of the three following single amino acid substitutions: Met-69→(Ile/Leu/Val), Arg-244→(Ser/Thr/Cys) or Asn-276→Asp. TEM-50[12] and SHV-10[13] are of great concern since they combine amino acid substitutions of ESBL and IR variants. Nevertheless, characterization of TEM-50 and SHV-10 implied that both enzymes appeared to be unable to confer a full ESBL phenotype.

In order to investigate the effects of amino acid substitutions mediating IR in TEM β-lactamases on the resistance phenotypes conferred by SHV β-lactamases, we introduced the substitutions at homologous positions within the SHV sequence.

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Materials and methods

Plasmids and bacteria

The low copy plasmid vector pCCR9[14] harbouring a tetracycline selection marker was used for cloning of various bla_{SHV} genes. *Escherichia coli* DH5α[15] was used as a recipient for transformation with recombinant plasmids. The strains MPB-1, MPB-2 and MPB-5, producing the parental β-lactamases SHV-1, SHV-2 and SHV-5, respectively, were taken from a panel of isogenic SHV producers described previously.[16]

Antibiotics

Tetracycline was obtained from Pfizer (Groton, CT, USA).

Oligonucleotides

The sequences of the oligonucleotides used for site-directed mutagenesis were the following: for Met-69→Ile (ATG→**ATT)**, 5′-GAACGCTTTCCCATG**ATT**AGCACCTTTAAAGTA-3′/3′-CTTGCGAAAGGGTAC**TA A**TCGTGGAAATTTCAT-5′; for Arg-244→Ser (CGC→**AGC**), 5′-CGGGGTGCG**AGC**GGGATTGTCGCCCTGCTTGGC-3′/3′-GCCCCACGC**TCG**CCCTAACAGCGGGACGAACCG-5′; and for Asn-276→Asp (AAT→**GAT)**; 5′-AGCATGGCCGAGCGA**GAT**CAGCAAATCGCCGGG-3′/3′-TCGTACCGGCTCGCT**CTA**GTCGTTTAGCGGCCC-5′. All oligonucleotides for site-directed mutagenesis (33-mers) and sequencing (20-mers) were custom synthesized by Microsynth (Balgach, Switzerland).

Antibiotic susceptibility testing

Disc agar diffusion testing was performed according to the guidelines of the NCCLS.[17] Etests (AB Biodisk, Solna, Sweden) were performed on plates containing a 4 mm layer of Mueller–Hinton agar (Difco, Detroit, MI, USA), according to the manufacturer's protocols.

DNA preparation

DNA of recombinant plasmids was prepared using the Qiagen plasmid kit (Qiagen, Hilden, Germany). The manufacturer's instructions were followed. Standard protocols were applied for the extraction of total DNA.[18]

Mutagenesis

The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to introduce single nucleotide exchanges into bla_{SHV} genes located on recombinant plasmids. These alterations were confirmed by sequencing as were the entire open reading frames and the 400 bp upstream and 300 bp downstream flanking regions. Genes that had received the correct modifications were re-cloned on 3.6 kb fragments into pCCR9 vector to prevent aberrations from complete isogenicity through possible undetected mistakes within the non-sequenced part occurring upon mutagenesis. Re-cloning was carried out using *Asp*718–*Sph*I restriction sites and the ligation products were transformed into *E. coli* DH5α for evaluation of resistance.

Re-cloning of bla_{SHV} genes and confirmation of sequence

Ten micrograms of vector or recombinant plasmid DNA were digested with appropriate restriction enzymes following the supplier's protocols (Hoffmann La Roche, Basel, Switzerland). After agarose gel electrophoresis, the linearized vector band and selected insert fragments were cut from the gel, extracted and purified by using the NucleoSpin Extrakt Kit (Machery-Nagel, Düren, Germany). Recircularization of vector was prevented by pretreatment with calf intestinal phosphatase (Hoffmann La Roche), and ligation was with T4 DNA ligase (Hoffmann La Roche) for 18 h at 4°C, according to the manufacturer's instructions. The ligation mixtures were used to transform competent *E. coli* DH5α cells according to the standard protocols of Sambrook *et al.*[18] Recombinants were picked and purified on LB agar plates (Difco) containing 10 mg/L tetracycline. The recombinant plasmids were checked for correct size and orientation of the insert by restriction mapping.[18]

DNA sequences were determined by the dideoxy nucleotide chain termination method[19] using an ABI Prism 310 Genetic Analyzer. The ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, CA, USA) was used according to the supplier's recommendations. Sequences were processed with the Auto Assembler, version 1.4.0 (Perkin-Elmer) and analysed with the GCG sequence analysis software package, version 9.0 (Genetics Computer Group, Madison, WI, USA).

Results

Construction of SHV-IR mutants by site-directed mutagenesis

Three amino acid substitutions, Met-69→Ile, Arg-244→Ser and Asn-276→Asp, were introduced by site-directed mutagenesis of the *bla* genes within three parental isogenic strains carrying either bla_{SHV-1}, bla_{SHV-2} or bla_{SHV-5}. They were introduced alone and in all possible combinations involving two or three mutations, until seven variants of each parental strain were established. Designation of the resulting clones was in accordance with the following examples: the strain expressing SHV-1 and carrying the substitutions Met-69→Ile and Arg-244→Ser was called ICB-1(Ile-69, Ser-244).

Decrease of resistance against β-lactam antibiotics

Etests were performed to obtain a resistance pattern for each mutant (Table I). The results obtained with the disc diffusion method were in good agreement with the Etest (data not shown). Comparison of the resistance of parental and mutant strains revealed that the new strain constructions generally expressed reduced resistance against non-combined β-lactam antibiotics. The ESBL phenotypes (defined by the MICs of expanded-spectrum cephalosporins and aztreonam) of the mutants derived from SHV-2 and SHV-5 were drastically reduced, resulting in resistance levels similar to those of the producer of the non-ESBL SHV-1 (Table I). This effect was particularly pronounced in mutants that contained two or three amino acid substitutions (Table I). As an exception, the substitution Asn-276→Asp exerted little or no impairment of resistance to these compounds in mutants derived from either SHV-2 or SHV-5, and cefepime resistance was even increased two- to three-fold. Single substitution at position 69 caused moderate reduction of resistance against the β-lactams tested, and the greatest reduction by a single change was observed through the substitution at position 244. Of the mutants with combinations of IR-specifying substitutions, those carrying Met-69→Ile+Asn-276→Asp clearly had the least diminishing effect. Their cephalosporin resistance, apart from a few exceptions, remained higher than those expressed by carriers of the single substitution at position 244. Combinations of the other substitutions, Arg-244→Ser+Asn-276→Asp, Met-69→Ile+Arg-244→Ser and Met-69→Ile+Arg-244→Ser+Asn-276→Asp, abolished resistance almost totally. The MICs of expanded-spectrum cephalosporins were hardly, if at all, above those reached by the control carrying the unaltered vector.

The same tendencies were observed while testing the MICs of the penicillins although interpretation was not always possible because many of the MICs were >256 mg/L.

Increase of resistance against β-lactamase inhibitors

Etests were performed to assess the IR phenotypes of the mutants (Table II). As expected, MICs of inhibitor/β-lactam combinations for the carriers of IR-specifying mutations were generally higher than those for the producers of the respective parental enzymes. This effect, however, was barely noticeable with ceftazidime/clavulanic acid (Table II). No effect was observed with cefotaxime/clavulanic acid (not shown), because all MICs were below the range covered by the respective Etest strips. In the non-ESBL SHV-1, introduction of Met-69→Ile alone or together with Asn-276→Asp, caused the strongest increase in resistance, two- to 43-fold (Table II), followed by the derivatives carrying the single substitutions Asn-276→Asp or Arg-244→Ser. Other double or triple substitutions barely lowered the MICs of inhibitor combinations (Table II).

In contrast, the derivatives of the ESBLs SHV-2 and SHV-5 exerted the strongest effects, with one- to eight-fold elevation of MICs of inhibitor combinations, when the substitution Arg-244→Ser alone or in combination with Met-69→Ile was introduced. The single substitution derivatives Asn-276→Asp and Met-69→Ile and the multiple substitution derivatives, Met-69→Ile+Asn-276→Asp, Arg-244→Ser+Asn-276→Asp and Met-69→Ile+Arg-244→Ser+Asn-276→Asp, generally had a minor and varying impact on resistance of the carrier to the inhibitor combinations, averaging between a two-fold increase and a two-fold decrease (Table II).

Only a few derivatives of the non-ESBL SHV-1, e.g. ICB-1(Ile-69), ICB-1(Ile-69, Asp-276) and, to a lesser extent, ICB-1(Asp-276), were able to mediate inhibitor resistance significantly above the lower NCCLS breakpoints for clinical relevance.[20] None of the combined IR–ESBL mutants reached MICs of inhibitors that were >32-fold above background (*E. coli* DH5α/pCCR9; Table II), and none of the IR–ESBL mutants, except ICB-5(Asp-276) and ICB-5(Ile-69, Asp-276), led to MICs of expanded-spectrum cephalosporins that were above the lower NCCLS breakpoint of 8 mg/L or even above the NCCLS ESBL screening breakpoint of 1 mg/L,[20] the vast majority remaining below 0.25 mg/L (Table I).

Absence of combined IR–ESBL phenotypes

The fold-increases of resistance to inhibitor combinations conferred by the IR–ESBL constructions, derived from SHV-2 and SHV-5, were compared with the corresponding fold-decreases of resistance to expanded-spectrum cephalosporins (Tables I and II). Some of the constructions, predominantly among those harbouring two or three substitutions, even suffered loss of activity within both phenotypes. However, the relative loss of expanded-spectrum cephalosporin resistance was greater than that of inhibitor resistance. Cumulative ranking of the data on cefotaxime, ceftriaxone, ceftazidime and cefepime for the IR–ESBL derivatives of SHV-2 and SHV-5 revealed that the substitution Asn-276→Asp caused the least impairment of resistance, followed by Met-69→Ile, Met-69→Ile+Asn-276→Asp, Arg-244→Ser, Met-69→Ile+Arg-244→Ser, Arg-244→Ser+Asn-276→Asp and Met-69→Ile+Arg-244→Ser+Asn-276→Asp in increasing order. The corresponding ranking based on the data for co-amoxiclav, ampicillin/sulbactam, piperacillin/tazobactam and cefoperazone/sulbactam, showed that Arg-244→Ser led to the strongest increase of inhibitor resistance, followed by Met-69→Ile+Arg-244→Ser, Asn-276→Asp, Met-69→Ile+Asn276→Asp, Arg-244→Ser+Asn-276→Asp, Met-69→Ile+Arg-244→Ser+Asn-276→Asp and Met-69→Ile in decreasing order.

As a general trend, we noted that a gain in inhibitor resistance came at the expense of expanded-spectrum

Table I. Susceptibilities of the mutants based on the pCCR9 vector against various β-lactam antibiotics

Strain	MICs (mg/L) by Etest												
	CEF	CXM	CTX	CAZ	CRO	FEP	CPD	CFP	ATM	AMX	AMP	PIP	TIC
CTB-1	8	0.25	0.008	0.094	0.008	0.047	0.125	3	0.016	>256	>256	>256	>256
ICB-1(Ile-69)	4	0.38	0.008	0.032	0.003	0.023	0.125	2	<0.016	>256	>256	>256	>256
ICB-1(Ser-244)	3	0.25	0.008	0.032	0.003	0.023	0.064	0.38	<0.016	>256	>256	1.5	>256
ICB-1(Asp-276)	8	0.25	0.008	0.19	0.004	0.047	0.094	0.75	0.016	>256	>256	>256	>256
ICB-1(Ile-69, Ser-244)	4	0.25	0.008	0.032	0.002	0.012	0.094	<0.016	<0.016	>256	32	8	6
ICB-1(Ile-69, Asp-276)	4	0.25	0.008	0.047	0.003	0.016	0.094	1.5	<0.016	>256	>256	>256	>256
ICB-1(Ser-244, Asp-276)	4	0.25	0.008	0.032	0.003	0.016	0.094	0.032	<0.016	>256	24	3	6
ICB-1(Ile-69, Ser-244, Asp-276)	4	0.19	0.008	0.023	0.002	0.012	0.064	<0.016	<0.016	8	4	1.5	1
CTB-2	>256	3	3	0.50	1	0.19	32	2	0.19	>256	>256	256	>256
ICB-2(Ile-69)	16	0.50	0.047	0.094	0.032	0.094	0.75	0.75	<0.016	>256	>256	32	>256
ICB-2(Ser-244)	4	0.38	0.008	0.032	0.002	0.064	0.094	0.75	<0.016	>256	>256	16	>256
ICB-2(Asp-276)	>256	1	0.5	0.50	0.25	0.38	8	1	0.064	>256	>256	256	>256
ICB-2(Ile-69, Ser-244)	3	0.19	0.008	0.023	0.002	0.016	0.064	0.064	<0.016	>256	128	12	64
ICB-2(Ile-69, Asp-276)	6	0.19	0.023	0.125	0.008	0.25	0.25	0.75	<0.016	>256	>256	48	>256
ICB-2(Ser-244, Asp-276)	2	0.125	0.008	0.023	0.002	0.032	0.094	0.125	<0.016	>256	96	4	8
ICB-2(Ile-69, Ser-244, Asp-276)	3	0.19	0.008	0.023	0.002	0.012	0.094	<0.016	<0.016	16	6	3	3
CTB-5	>256	8	8	24	4	0.25	64	8	16	>256	>256	>256	>256
ICB-5(Ile-69)	48	0.38	0.094	0.75	0.19	0.094	2	1	0.25	>256	>256	48	>256
ICB-5(Ser-244)	4	0.25	0.016	0.094	0.006	0.19	0.19	1	1.5	>256	>256	64	>256
ICB-5(Asp-276)	>256	1.5	2	48	1.5	0.75	24	1.5	8	>256	>256	>256	>256
ICB-5(Ile-69, Ser-244)	3	0.25	0.008	0.047	0.002	0.023	0.094	0.50	<0.016	>256	>256	16	>256
ICB-5(Ile-69, Asp-276)	16	0.38	0.047	3	0.047	0.38	1	1	0.19	>256	>256	64	>256
ICB-5(Ser-244, Asp-276)	3	0.25	0.008	0.023	0.003	0.012	0.094	<0.016	<0.016	1.5	0.75	0.25	0.5
ICB-5(Ile-69, Ser-244, Asp-276)	4	0.19	0.008	0.016	0.002	0.023	0.094	0.023	<0.016	32	12	4	48
ECDH5α(pCCR9)	2	0.38	0.008	0.023	0.008	0.012	0.094	<0.016	<0.016	1.5	0.75	0.19	0.5
ECDH5α	4	0.50	0.008	0.023	0.002	0.008	0.094	<0.016	<0.016	1.5	0.75	0.19	0.5

AMP, ampicillin; AMX, amoxicillin; ATM, aztreonam; CAZ, ceftazidime; CEF, cephalothin; CFP, cefoperazone; CPD, cefpodoxime; CRO, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; FEP, cefepime; PIP, piperacillin; TIC, ticarcillin.

Table II. Susceptibilities of the mutants based on the pCCR9 vector against β-lactam antibiotics/β-lactamase inhibitor combinations

Strain	MICs (mg/L) by Etest					
	AMC	TIM	SAM	TZP	TZL	CPS
CTB-1	4	8	64	0.75	<0.064	0.75
ICB-1(Ile-69)	16	>256	192	24	<0.064	1.5
ICB-1(Ser-244)	4	2	16	1	<0.064	0.19
ICB-1(Asp-276)	8	16	96	4	<0.064	0.50
ICB-1(Ile-69, Ser-244)	4	1.5	12	4	<0.064	0.023
ICB-1(Ile-69, Asp-276)	16	>256	128	32	<0.064	1
ICB-1(Ser-244, Asp-276)	3	1	6	0.75	<0.064	0.023
ICB-1(Ile-69, Ser-244, Asp-276)	4	0.75	1.5	1	<0.064	<0.016
CTB-2	3	1.5	1.5	0.19	<0.064	0.25
ICB-2(Ile-69)	1.5	1.5	2	0.19	<0.064	0.094
ICB-2(Ser-244)	8	2	8	0.5	<0.064	0.25
ICB-2(Asp-276)	4	1.5	3	0.19	<0.064	0.25
ICB-2(Ile-69, Ser-244)	6	3	8	1.5	<0.064	0.064
ICB-2(Ile-69, Asp-276)	4	1.5	3	0.25	<0.064	0.094
ICB-2(Ser-244, Asp-276)	4	1.5	6	0.75	<0.064	0.064
ICB-2(Ile-69, Ser-244, Asp-276)	2	1	1.5	1.5	<0.064	<0.016
CTB-5	2	2	1.5	0.19	<0.064	0.19
ICB-5(Ile-69)	3	3	1.5	0.19	0.094	0.064
ICB-5(Ser-244)	6	12	4	0.25	<0.064	0.19
ICB-5(Asp-276)	3	3	1.5	0.25	0.064	0.19
ICB-5(Ile-69, Ser-244)	2	16	4	0.5	0.064	0.19
ICB-5(Ile-69, Asp-276)	3	4	2	0.19	0.125	0.064
ICB-5(Ser-244, Asp-276)	1	0.5	0.5	0.125	<0.064	<0.016
ICB-5(Ile-69, Ser-244, Asp-276)	4	6	2	0.75	<0.064	0.032
*EC*DH5α (pCCR9)	1	0.5	0.5	0.19	<0.064	<0.016
*EC*DH5α	1	0.38	0.38	0.38	<0.064	<0.016

AMC, co-amoxiclav; SAM, ampicillin/sulbactam; CPS, cefoperazone/sulbactam; TZP, piperacillin/tazobactam; TIM, ticarcillin/clavulanic acid; TZL, ceftazidime/clavulanic acid.

cephalosporin resistance. Consequently, none of the hybrid IR–ESBL SHV enzymes alone (Tables I and II) was able to mediate simultaneous resistance to both expanded-spectrum cephalosporins and inhibitor/β-lactam combinations. Moreover, even the highest MICs of inhibitor combinations mediated by these hybrid enzymes were only at moderate levels. This was probably owing to the general loss of hydrolytic activity towards all β-lactams including penicillins (Table I). Such an effect of mutual compensation by the two types of substitution were not seen with the mutants derived from non-ESBL SHV-1. Consequently, these mutants showed the highest levels of resistance to inhibitor combinations (Table II).

Discussion

Ambler class A β-lactamases, belonging to subgroups 2b, 2be, 2br and 2f, inactivate all major classes of β-lactam antibiotics.[1] However, ESBLs and IR variants are presently of greatest concern due to their worldwide occurence and difficulty in their detection.[2,3,21,22] Moreover, because inhibitor/β-lactam formulations are recommended options for therapy of ESBL-producing pathogens,[6,7,23,24] the potential evolution through homologous recombination, of hybrid enzymes expressing both the ESBL and the IR phenotype is of particular concern. Such a scenario can be anticipated, since (i) both ESBL and IR variants arise through single amino acid substitutions at the active site, and (ii) carriage of multiple *bla* genes within single host strains is common.[25,26] Indeed, chimeric enzymes (e.g. TEM-50 and SHV-10) have been discovered in clinical isolates,[12,13] but have shown serious impairment of their ESBL phenotype so far. Consequently, catalytic and phenotypic activities of IR–ESBL constructions of TEM and SHV enzymes were analysed.[27–33] Moreover, a systematic analysis of mutationally derived IR-TEM–ESBL variants has been reported recently.[34]

We present the results of a systematic study on the phenotypic effect of amino acid substitutions typical for IR-TEM enzymes, when introduced at the homologous and otherwise conserved positions of selected non-ESBL and ESBL SHV β-lactamases in an isogenic genetic background. The isogenic background crucial for accurate evaluation of mutational influences is warranted through a system established previously[16] and refined recently.[14]

The most important finding of the present study is that none of the 14 SHV-IR–ESBL derivatives was able to confer high-level resistance to both expanded-spectrum cephalosporins and inhibitor/β-lactam combinations. This was found even when all seven possible combinations of the three TEM-type inhibitor-determining substitutions were systematically introduced into the two most important types of SHV ESBL: (i) SHV-2 carrying the most abundant alteration, Gly-238→Ser; and (ii) SHV-5 featuring Gly-238→Ser and Glu-240→Lys, the latter being responsible for a boost of ceftazidime resistance.[14,35] These results confirm and extend the findings of others, who worked with IR–ESBL constructions.[28–30,33,34,36] Moreover, they reveal that the levels of resistance to both expanded-spectrum cephalosporins and inhibitor combinations of the SHV-IR–ESBL derivatives were generally lower than those of the respective TEM derivatives. This may partly explain why the three TEM-specific IR substitutions have not been found in clinical SHVs so far, and why far fewer SHV than TEM variants have been discovered to date.

A second important finding is that the SHV derivatives generally did not benefit from accumulation of multiple IR-type substitutions, since in most cases both the ESBL and the IR phenotypes conferred were drastically reduced. This observation, which is consistent with an earlier report on TEM derivatives,[37] may reflect a destabilizing effect of mutations on the enzyme structure. This may result in reduced activity and/or a reduction in the amount of functional protein in the periplasm, leading to reduced resistance. The only partial exceptions were Met-69→Ile +Asn-276→Asp, which caused only moderate impairment of the ESBL phenotype, and Met-69→Ile+Arg-244→Ser, which resulted in the second highest overall inhibitor resistance. Despite these partial exceptions, it is reasonable to conclude that no IR–ESBL synergy between any of the three IR substitutions will occur through homologous recombination.

The fact that resistance to inhibitors and extended-spectrum cephalosporins are indirectly proportional when the MICs for all SHV-IR–ESBL derivatives are analysed suggests that the two phenotypes are mutually exclusive. This hypothesis is supported by the observation that TEM-ESBLs challenged with inhibitor combinations have been shown to revert back to TEM-1 rather than to evolve to IR-TEM–ESBLs.[38] Similarly, a strain expressing an IR-TEM enzyme exposed to a single β-lactam rather than IR-TEM–ESBLs produced TEM-1 revertants.[39] This has prompted some workers to call for more formulations combining later-generation cephalosporins with inhibitors.[7] Our data strongly support this strategy, since none of our SHV-IR–ESBL constructions showed reduced susceptibility to cefoperazone/sulbactam or ceftazidime/clavulanate (Table II). Indeed, in the latter case, the MICs for most derivatives remained below the limit of detection of the respective Etest strips.

Further interesting new aspects were noted. In our system, modification of position 244 had the most profound effect on augmentation of inhibitor resistance in both SHV-ESBLs used. In contrast, TEM-ESBLs gained the greatest lR increase when positions 69 and 276 were altered.[34] Two factors could potentially explain this difference: (i) distinct structural properties at the active site of the TEM and the SHV structure; or (ii) the introduction of Arg-244→Ser as opposed to Arg-244→Cys replacements realized in the TEM system. Although both effects may be important, the intrinsic structural differences between TEM- and SHV-ESBLs seem to have a predominant effect, as in our SHV system, the non-ESBL variants behaved exactly as the non-ESBL IR-TEM variants, with Met-69→Ile and Met-69→Ile plus Asn-276→Asp increasing inhibitor resistance most and Arg-244→Ser ranking only fourth out of seven (Table II). This interpretation is also supported by Bret and co-workers,[27] who found only minor differences when they compared the effects of either Ser, Cys or His as replacements for Arg-244. In this context, it is also worth mentioning that although Arg-244→Ser alone or in combination with Met-69→Ile had the greatest impact on the increase in inhibitor resistance in SHV-IR–ESBL variants, this rise was only one- to eight-fold, and was accompanied by an up to 2000-fold decrease of expanded-spectrum cephalosporin resistance (Tables I and II). Interestingly, and consistent with our results, OHIO-1, an non-ESBL SHV enzyme very closely related to SHV-1, also gained little inhibitor resistance by Arg-244→Ser, or became even more susceptible to certain combinations including ampicillin/sulbactam and piperacillin/tazobactam while Met-69→Ile led to broad-spectrum inhibitor resistance.[31,32] The reason Arg-244→Ser behaves so differently within the SHV-IR–ESBLs compared with within the SHV-IR–non-ESBL is not known.

Considering that substitution Asn-276→Asp alone caused almost no weakening of resistance to single β-lactams on the one hand, and an only 1.5- to five-fold increase of inhibitor resistance on the other (Tables I and II), it is clearly the alteration with the least influence. This observation is not surprising since it reflects the relatively unimportant role that this change plays in the TEM background, where it has never been found alone but always in concert with a change at position 69.[11] Comparing our data with those reported by Stapleton and co-workers,[34] it is obvious that the TEM derivatives confer increased co-amoxiclav resistance and remain fully susceptible to piperacillin/tazobactam, while the opposite is true for the hybrid SHV variants. This suggests that the active sites of

hybrid TEM and SHV enzymes may be differentially accessible for clavulanate and tazobactam.

In conclusion, SHV β-lactamases will not necessarily benefit from recombination events that unify substitutions leading to the IR and the ESBL phenotype. If a hybrid IR–ESBL phenotype does occur, it is likely to be even weaker than that mediated by an analogous IR-TEM–ESBL derivative. In the case of the inhibitor tazobactam only, hybrid SHVs may be superior. These findings are reassuring for clinicians and clinical microbiologists who are concerned about the possible evolution of hybrid IR–ESBL enzymes. However, some caution is justified because compensatory substitutions away from the critical positions identified thus far, might lead to a β-lactamase that is able simultaneously to confer high-level resistance to both expanded-spectrum cephalosporins and inhibitor/β-lactam combinations. A structure of this kind has been presented at a recent meeting but has not as yet been described in full.[40]

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