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THE AMPLIFIED CHEMILUMINESCENCE TEST TO CHARACTERIZE ANTIRHEUMATIC DRUGS AS OXYGEN RADICAL SCAVENGERS*

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Abstract—High levels of reactive oxygen species (ROS) are generated by phagocytes involved in host defence and inflammation. Thus, it appears highly desirable to learn more about the potential of antirheumatic drugs to scavenge ROS or to inhibit their enzymatic generation.

Amplified chemiluminescence (CL) allows detection of O_2^- using lucigenin (LgCL) or H_2O_2 using luminol (LuCL). A total of 43 compounds have been tested quantitatively *in vitro* (10^{-6} to 10^{-4} mol/l) with respect to three test parameters; varying cell-activity, and incubation-time employing two different phagocyte populations (neutrophils/macrophages).

The most active compounds with LgCL were the known radical scavengers nordihydroguaiaretic acid (NDGA), *N*-propyl gallate, superoxide dismutase and chloroquine, the non-steroidal anti-inflammatory drugs (NSAID) benzydamine, timegadine, carprofen, enolicam, the known lipoxygenase inhibitors (e.g. CBS 1108/1114, BW 755C) and glucosaminoglucan polysulfate. Inactive in this system were corticosteroids (prednisolone, dexamethasone) most of the tested NSAID (N = 16/20), most disease modifying drugs (D-penicillamine, levamisole, gold-TM) and the anti-gout drugs (sulfinpyrazone, allopurinol, colchicine). Therefore amplified CL with lucigenin appears to be a rapid, kinetic, reproducible means of pharmacological profiling *in vitro* new anti-inflammatory drugs for radical scavenger activity.

Phagocytes generate reactive oxygen species (ROS)‡ which can be both beneficial and destructive to the host (1). Together with lysosomal enzymes, ROS contribute considerably to various immune and non-immune inflammatory conditions and may even promote carcinogenesis [2, 3].

Only phagocytes appear to possess the membrane-bound NAD(P)H oxidase which reduces molecular oxygen to the superoxide anion radical O_2^-) [4]. The hexose monophosphate shunt delivers the redox equivalents for the NAD(P)H oxidase and appears also responsible for proton secretion [5]. The O_2^- radical dismutases spontaneously or enzymatically via the superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). O_2^- and H_2O_2 directly or catalyzed by Fe^{2+} lead to the highly tissue damaging hydroxyl radical ($OH^{\cdot}$). ROS are also generated during physiologic oxidation of arachidonic acid [6, 7].

The amount of O_2^- generated by phagocytes depends on various biological activators (ligands) reacting with specific cell receptors, like immune complexes (Fcγ or complement C3b receptors), complement anaphylatoxin C5a, the self-activating mediators as leukotriene B_4 and the platelet activating factor [8]. This is important, because pharmacological effects are highly dependent upon the nature of the stimulus [9–12].

The phagocyte activation via the antibody and/or complement mediated mechanisms is at present hardly influenced pharmacologically [13]. Generally, antirheumatic drugs are biochemically characterized by their inhibitory properties towards the arachidonate metabolizing enzymes cyclo- and lipoxygenase [14]. The most often used non-steroidal anti-inflammatory drugs (NSAID) inhibit the cyclooxygenase, but do not effectively prevent leukocyte activation and the degranulation of lysosomal enzymes [9, 15–18]. In the past, many biochemical events during phagocyte activation have been studied for possible relevance for *in vitro* inflammatory pharmacology but failed [19]. *In vitro* pharmacology with phagocytes is complicated by the fact that various phagocyte functions often run parallel, but the signal coupling is still unclear [20].

The chemiluminescence (CL) test records phagocyte activity via native (low level) or chemically amplified photon emission evoked by ROS [21]. CL has become a new kinetic clinical and laboratory routine procedure [22, 23]. Amplified CL employs the chemicals luminol or lucigenin which react largely with H_2O_2 or O_2^-, respectively [24]. Although both tests have been used for *in vitro* pharmacological

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‡ Abbreviations used: AA, arachidonic acid; CL, chemiluminescence; ZyC3b, complement-opsonized zymosan; DMSO, dimethylsulfoxide; FMLP, formyl-methionyl-leucyl-phenylalanine; LgCL, lucigenin-amplified CL; LuCL, luminol-amplified CL; MØ, macrophages; NBT, nitroblue tetrazolium reduction test; NSAID, non-steroidal anti-inflammatory drugs; NDGA, nordihydroguaiaretic acid; PEC, peritoneal exudate cells; PMNL, polymorphonuclear leukocytes; ROS, reactive oxygen species; SOD, superoxide dismutase.

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purposes [25–27], a thorough comparison does not exist. Indeed, it is already recognized that both amplifiers give different pharmacological results [24, 28]. The most often applied photometric tests to determine ROS generation *in vitro* are the nitroblue tetrazolium reduction test (NBT) and ferricytochrome *c* reduction, the latter determining O_2^- [29]. Both tests are endpoint determination methods. The NBT is less sensitive than LuCL [30].

It will be shown that except for some few drugs most of the tested NSAID do not interfere with O_2^--mediated LgCL or H_2O_2-mediated LuCL. The radical scavenging properties of the physiologic ROS protectants catalase and SOD are compared with some known chemicals, the new class of lipoxygenase inhibitors and disease modifying drugs. LgCL was found to be preferable to LuCL for routine *in vitro* pharmacology.

MATERIALS AND METHODS

Compounds. The following compounds are used: allopurinol (Sigma Chemical Co., München, F.R.G.), arachidonic acid and arachidonic acid sodium salt (Sigma Chemical Co.), acetylsalicylic acid (E. Merck, Darmstadt, F.R.G.), benoxaprofen (Eli Lilly GmbH, Bad Homburg, F.R.G.), benzydamine (Giulini Pharma GmbH, Hannover, F.R.G.), bufexamac (Sigma Chemical Co.), BW 755C × HCl (Wellcome Research Laboratories, Beckenham, U.K.), carprofen (Hoffmann-La Roche, Grenzach, Switzerland), catalase (Sigma Chemical Co.), CBS 1108 oxalate and CBS 1114 HCl salt (Chauvin Blache, Montpellier, France), chloroquine (Sigma Chemical Co.), colchicine (Sigma Chemical Co.), dexamethasone (Sigma Chemical Co.), diclofenac (Ciba-Geigy GmbH, Wehr, F.R.G.), dimethylsulfoxide (DMSO) (E. Merck), enolicam (Ciba-Geigy GmbH), ebselen (A. Nattermann, Köln, F.R.G.), etofenamate (Troponwerke GmbH, Köln, F.R.G.), flufenamic acid (Kali-Chemie Pharma, Hannover, F.R.G.), GAGPS (glucosaminoglucan polysulfate) (Luitpold, München, F.R.G.), gold thiomalate (TM) (EGA-Chemie, Steinheim, F.R.G.), ibuprofen (Boots Co. Ltd., Nottingham, U.K.), indomethacin (Arbochem, Milano, Italy), ketoprofene (Bayer AG, Leverkusen, F.R.G.), levamisole (Janssen, Neuss, F.R.G.), metamizole (Hoechst, Frankfurt, F.R.G.), naproxen (Seci-Pharma, Milano, Italy), nordihydroguaiaretic acid (NDGA) (Sigma Chemical Co.), D-penicillamine (Fluka, Neu-Ulm, F.R.G.), phenidone (Pfannenschmidt, Hamburg, F.R.G.), piroxicam (Sigma Chemical Co.), pirprofen (Ciba-Geigy GmbH), prednisolone hemisuccinate (Sigma Chemical Co.), *N*-propyl gallate (Sigma Chemical Co.), retinol (Sigma Chemical Co.), sulfasalazine (Sigma Chemical Co.), sulfinopyrazone (Ciba-Geigy GmbH), sulindac (Merck-Sharp-Dome, Rahway, NY), superoxide dismutase (Sigma Chemical Co.), timegadine (Leo, Ballerup, Denmark). All compounds with quantitative determination (10^{-6} to 10^{-4} mol/l), without solvent in Dulbecco's phosphate buffered saline.

Peritoneal exudate cells (PEC). PEC are induced by injection of thioglycollate (3%, 2 ml; fluid, dehydrated, Difco Laboratories, U.S.A.) in sterile saline into female inbred BALB/cAnNCr1BR mice (Charles River Wiga GmbH, Sulzfeld, F.R.G.), 20–25 g body weight. The cells are harvested as two independent cell pools from four animals per day each. Cells harvested 24 hr after injection of thioglycollate are mainly polymorphonuclear leukocytes (PMNL) or harvested 96 hr thereafter are mainly macrophages (MØ) [31] and were adjusted to 2×10^6 cells/ml with RPMI 1640 (Flow Laboratories, Meckenheim, F.R.G.) containing 10% fetal calf serum (FCS; Seromed GmbH, München, F.R.G.), substituted with 18 μmol/l sodium bicarbonate (Flow Laboratories), pH 7.2, and L-glutamine 2 μmol/l (Flow Laboratories). PEC are kept on ice till use.

Chemiluminescence measurement. Experiments are performed according to a previously published method [24], yet at present using a luminescence analyzer Auto-Biolumat LB 950 (Laboratorium Prof. Dr. Berthold, Wildbad, F.R.G.). Major technical adaptations were done in respect to chain length, modification of the incubation chamber, and fully computerized (Apple IIe) injection of the amplifier and cell activator. The CL amplifier lucigenin (Sigma Chemical Co., München, F.R.G.); 1.54×10^{-4} mol/l final concentration, and luminol (1.54×10^{-4} mol/l; Lumanol-100 R, Lumac, Abimed Analysen-Technik GmbH, Düsseldorf, F.R.G.) are used. Cells are activated by human complement-opsonized zymosan (Sigma Chemical Co., München, F.R.G.) (ZyC3b: 1.6 mg/ml final concentration; preparation according to [31], suspended in phosphate-buffered saline (Dulbecco's modification with Ca^{2+} and Mg^{2+}; Flow Laboratories).

Peak CL starts with a 10 min adaptation period of 100 μl cell suspension at 37° after which 100 μl of the amplifier is added. The pre-peak period consists of two determination points (6 sec each with an 8 min interval). Cells are activated by adding 100 μl ZyC3b and additionally 100 μl DPBS. After a 16 min peak period with two determination points, 100 μl of the test compound are added, and during the total incubation time of 16 min with the compound two further determination points with an interval of 8 min are recorded.

The next two parameters follow the same test principle yet after a 30 min preincubation period preceded by a 5 min adaptation time for the cells starting with 100 μl cell suspension plus 100 μl compound solution. Thereafter, 100 μl of the amplifier are added and for a period of 16 min with three determination points the parameter spontaneous CL is determined. Thereafter, the activator is added automatically (100 μl ZyC3b plus 100 μl DPBS) and the parameter induced CL is determined during the final 24 min incubation period by three determination points with the total incubation time of 70 min and a final test volume of 0.5 ml. All determinations are duplicates. Spontaneous and induced CL are performed twice at the same day employing two separate cell populations.

Results are expressed as per cent inhibition or stimulation of the control sample (mean cpm with compound ×100/mean cpm without compound). Data acquisition and mathematical calculation based

on the integral of cpm detection includes the comparison of the two separate runs with the pre-incubation period (exclusion of data above 50% difference) with mean of duplicates, fully computerized print-out of the final protocols and computerized calculation of IC_{25} and IC_{50} values by best fit of the regression line according to the least square method (N = 42, except timegadine).

RESULTS

(1) *Comparison of test parameters*

Peak CL is the least sensitive but best reproducible test parameter (e.g VC 14%, N = 7 with aurintricarboxylic acid 24 hr PEC LgCL) due to the short incubation time of 16 min with maximally activated cells.

The overall test sensitivity is enhanced by a 30 min preincubation period of resting cells at 37°. Thereafter, spontaneous CL is determined for 16 min. In addition to the higher concentration of the test compound 1.7×10^{-4} mol/l with unstimulated cells spontaneous CL demonstrates the highest CL inhibition with both amplifiers, yet the poorest reproducibility. Therefore, two separate cell-pools are employed daily. The succeeding induced CL is better reproducible than spontaneous CL but less sensitive. Differences between induced CL and peak CL are considered to reflect largely time-dependent compound effects (16 versus 70 min incubation time).

The combination of 96 hr PEC with LgCL gives the best correlation of parameters ($r = 0.71$–0.89), the poorest LuCL with 24 hr PEC ($r = 0.26$ spontaneous CL versus peak CL). Results are best correlated by peak versus induced CL (r from 0.77 up to 0.91).

(2) *Comparison of cells*

The PMNL-rich 24 hr and MØ-rich 96 hr PEC give largely similar results with each of the three tested parameters (correlated compounds N = 42). The best correlation demonstrates peak CL with both amplifiers (LgCL $r = 0.75$; LuCL $r = 0.86$) and two different cell populations (24 hr, 96 hr). Thus, the most reliable parameter is again peak CL, followed by induced and spontaneous CL.

(3) *Comparison of the amplifiers lucigenin (Lg) and luminol (Lu)*

The different sensitivity of Lg and Lu to ROS is reflected by some opposite results with certain compounds (Table 1) demonstrated by a low correlation coefficient for LgCL/LuCL using either 24 hr or 96 hr PEC (poorest correlation for 24 hr PEC peak CL/spontaneous CL $r = 0.28$; best correlation peak CL $r = 0.64$). Again, peak CL and induced Cl show the most comparable results.

LgCL is preferred because it is most probably more related to cell activity and less dependent on the H_2O_2 disposing enzymes catalase, glutathione peroxidase and myeloperoxidase than LuCL [23].

(4) *Comparison of tested compounds (Tables 1, 2)*

Results from 96 hr PEC are excluded from further consideration because they give similar results with most compounds as do 24 hr PEC but require more forward looking laboratory planning than do 24 hr PEC

Compounds resulting in comparable inhibition of ROS generation by 24 hr PEC with LgCL and LuCL (difference smaller 10%): NDGA, *N*-propyl gallate, sulfasalazine, GAGPS, carprofen, enolicam, sulfinopyrazone, timegadine.

Compounds resulting in divergent inhibition of ROS generation by 24 hr PEC with LgCL and LuCL (peak CL): (a) with LgCL 30% inhibition greater than LuCL: benzydamine—HCl, SOD, chloroquine. (b) with LgCL at least 18% inhibition lower than LuCL: catalase, CBS 1114, BW 755C-HCL, phenidone, CBS 1108, metamizol, allopurinol, indomethacin, phenylbutazone, diclofenac.

The most inhibitory compounds are the antioxidants NDGA and *N*-propyl gallate, the physiologic ROS protectants SOD and catalase, some lipoxygenase inhibitors (CBS 1114, sulfasalazine, BW 755C) and GAGPS. Among the tested NSAID benzydamine, timegadine, carprofen and enolicam are most active. Benzydamine exerts a similar preferential inhibition of LgCL as shown by SOD. Catalase expectedly inhibits more marked H_2O_2-dependent LuCL and preferentially with activated phagocytes. Nearly ineffective are 16 of the 20 tested NSAID and the corticosteroids (Table 1). A surprising similar inhibition irrespective of the CL parameters, cells and amplifiers is shown by sulfasalazine (not all data shown, Table 1).

(5) *Possible time-dependent compound effects*

Peak and induced CL record ROS-mediated photon emission both with maximally activated phagocytes, but dissimilar incubation time (16 versus 70 min). Differences in compound effects are most probably time-dependent. Compounds with stronger inhibitory effects (difference at least 10%) on induced CL than peak CL are: catalase, chloroquine, GAGPS, enolicam, CBS 1114, BW 755C, timegadine, flufenamic acid, arachidonic acid. Yet most tested compounds give similar results for peak and induced CL with LgCL ($r = 0.91$).

(6) *Compounds exerting CL enhancing effects*

These are often found with NSAID (Table 1) using LgCL for more than one test parameter (diclofenac, metamizole, bufexamac) and also with the arachidonic acid Na-salt and D-penicillamine.

LuCL is especially enhanced by some compounds (diclofenac, SOD, benzydamine) with unstimulated phagocytes, i.e. spontaneous CL.

Activation within the 24 hr PEC LgCL system is seen for *peak CL* (activation >20%) with bufexamac and arachidonic acid Na-salt, for spontaneous CL (activation >20%) with metamizole, D-penicillamine, colchicine, dexamethasone, diclofenac, arachidonic acid Na-salt and for *induced CL* (activation >20%) with arachidonic acid Na-salt only.

DISCUSSION

Lucigenin (Lg)- and luminol (Lu)-amplified chemiluminescence (CL) measure the generation of different reactive oxygen species (ROS) of phagocytes [24]. Therefore, both CL amplifiers may yield

Table 1. Lucigenin-/luminol-amplified chemiluminescence (CL) with 24 hr PMNL-rich peritoneal exudate cells (PEC) (% inhibition)

Parameter: Incubation time:	Peak CL (16 min)	Spontaneous CL (46 min) (30 min preincubation time included)	Induced CL (70 min)	
Amplifier: Compound (10^{-4} mol/l)	Lg (Lu)	Lg (Lu)	Lg (Lu)	
1. *NSAID* (largely cyclooxygenase inhibitors)				
Benzydamine-HCL	89 (49)	99 (+16)	99 (59)	
Timegadine	12 (17)	77 (64)	37 (63)	also ad 3
Carprofen	21 (20)	71 (73)	0 (20)	
Enolicam	20 (20)	53 (26)	43 (37)	also ad 3
Ibuprofen	3 (4)	36 (28)	6 (18)	
Indomethacin	+7 (39)	25 (67)	8 (44)	
Sulindac	9 (23)	22 (63)	5 (22)	
Phenylbutazone	1 (35)	20 (45)	1 (35)	
Benoxaprofen	+8 (+9)	18 (+5)	+2 (+36)	also ad 3
Ebselen	0 (+1)	16 (21)	4 (7)	also ad 3
Piroxicam	+8 (10)	13 (26)	+2 (14)	
Ketoprofen	5 (6)	8 (32)	+8 (14)	
Etofenamat	+5 (+8)	6 (46)	+2 (2)	
Naproxen	3 (15)	5 (43)	+11 (16)	
Pirprofen	+3 (17)	3 (44)	+14 (23)	
Bufexamac	+22 (13)	2 (68)	+14 (13)	
Acetyl salicylic acid	+7 (4)	+2 (+2)	1 (15)	
Flufenamic acid	+7 (+7)	+7 (29)	30 (+7)	
Metamizole	+16 (72)	+24 (68)	+14 (72)	
Diclofenac-Na	+3 (33)	+66 (+183)	4 (22)	
2. *Corticosteroids*				
Prednisolone hemisuccinate	+2 (+3)	13 (45)	+1 (2)	
Dexamethasone	16 (8)	+38 (3)	20 (22)	
3. *Lipoxygenase inhibitors*				
CBS 1108	4 (89)	78 (98)	3 (83)	
Sulfasalazine	55 (51)	56 (71)	56 (57)	also ad 4
CBS 1114	32 (97)	52 (96)	42 (95)	
BW 755C-HCl	27 (92)	34 (97)	38 (91)	
4. *Disease modifying drugs*				
Chloroquine	49 (11)	97 (68)	85 (42)	
Gold-TM	+3 (8)	40 (37)	+1 (13)	
Levamisole	+5 (6)	26 (50)	7 (9)	
D-Penicillamine	+8 (+16)	+29 (43)	5 (+13)	
5. *Antiarthrotics*				
GAGPS (2 mg/ml)	28 (29)	79 (83)	60 (75)	
SOD (40 μg/ml)	79 (47)	58 (+63)	78 (8)	also ad 7
6. *Anti-gout drugs*				
Sulfinpyrazone	17 (24)	21 (24)	8 (28)	
Allopurinol	5 (50)	+6 (31)	+1 (+2)	
Colchichine	10 (+3)	+32 (41)	8 (9)	
7. *Laboratory tools/antioxidants*				
NDGA	99 (99)	99 (99)	99 (99)	also ad 3
N-propyl gallate	97 (95)	97 (89)	97 (94)	also ad 3
Phenidone	11 (92)	45 (82)	13 (80)	also ad 3
Arachidonic acid	+12 (11)	32 (56)	27 (37)	
Catalase (20 mg/ml)	80 (98)	28 (+13)	91 (99)	
DMSO	3 (2)	15 (25)	6 (14)	
Retinol	+7 (7)	7 (39)	1 (+10)	
Arachidonic acid-Na	+300 (13)	+82 (73)	+105 (53)	

Exact assay conditions and calculations procedures are referred at Materials and Methods.

Table 2. Inhibitory concentrations (IC) of the most active compounds with respect to the Lg-amplified CL. ROS-generation by granulocyte-rich mouse peritoneal exudate cells (24 hr PEC)

Compound	Chemiluminescence parameter (LgCL)					
	Peak		Spontaneous		Induced	
	IC_{25} (mol/l)	IC_{50} (mol/l)	IC_{25} (mol/l)	IC_{50} (mol/l)	IC_{25} (mol/l)	IC_{50} (mol/l)
NDGA	4×10^{-6}	2×10^{-5}	8×10^{-7}*	5×10^{-6}*	2.3×10^{-6}	1×10^{-5}
N-propyl gallate	2.4×10^{-6}	8.9×10^{-6}	2.6×10^{-6}	1.1×10^{-5}	2.5×10^{-6}	9.4×10^{-6}
chloroquine	3.4×10^{-5}	—	2.7×10^{-6}	1×10^{-5}	9.5×10^{-6}	4.1×10^{-5}
CBS 1108	5×10^{-4}*	—	3.7×10^{-6}	2×10^{-5}	1×10^{-3}*	—
benzydamine	1×10^{-5}*	9×10^{-5}*	5×10^{-6}	1.7×10^{-5}	9×10^{-6}*	1×10^{-5}*
timegadine	—	—	8×10^{-6}*	5×10^{-5}*	5×10^{-5}*	4×10^{-4}*
sulfasalazine	3×10^{-5}*	1×10^{-4}*	1×10^{-5}*	9×10^{-5}*	3×10^{-5}*	9×10^{-5}*
BW 755C × HCl	—	—	1.2×10^{-5}*	—	1.8×10^{-5}*	—
carprofene	1.5×10^{-4}*	1×10^{-3}*	2×10^{-5}*	8×10^{-5}*	2×10^{-4}*	—
enolicam	5×10^{-4}*	—	5×10^{-5}*	2×10^{-4}*	5×10^{-5}*	2×10^{-4}*
SOD	10–20 μg/ml*	10–20 μg/ml*	<10 μg/ml*	<10 μg/ml*	<10 μg/ml*	<10 μg/ml*
GAGPS	1.8 mg/ml	—	0.04 mg/ml*	0.28 mg/ml	0.12 mg/ml*	1.75 mg/ml*
catalase	2.66 mg/ml	5.58 mg/ml	26.54 mg/ml	>40 mg/ml*	5 mg/ml	8.04 mg/ml

* Calculated from graph.

different results with various antirheumatic drugs interfering with ROS generation.

LgCL reacts mainly with O_2^- which is the first primary ROS generated by a phagocyte specific membrane-bound NADPH oxidase [4]. Thus, because LgCL most probably reflects phagocyte activity and because O_2^- appears of greater biological relevance than H_2O_2, LgCL is preferable. This is substantiated by the fact that for 43 tested compounds there was a better correlation of test parameters and phagocyte populations for LgCL rather than LuCL.

LuCL detects mainly H_2O_2 which is dependent on O_2^- generation, dismutation and H_2O_2 disposing and consuming enzymes as catalase, glutathione peroxidase (GPO) and myeloperoxidase (MPO) [24, 32]. The co-oxygenation reaction of luminol by the prostaglandin synthetase is of possible significance for LuCL and also the direct interference of drugs with the oxidation of luminol by H_2O_2 [26]. Until now no similar reactions with lucigenin have been published. These facts might also explain the poorer overall correlation found for LuCL compared to LgCL.

Three routinely determined test parameters refer to *in vitro* phagocyte activity and incubation time, i.e. (1) peak CL (16 min) with stimulated cells, (2) after 30 min preincubation spontaneous CL (46 min) with unstimulated cells and (3) induced CL (70 min) with activated cells. Of these three the most reliable yet least sensitive was peak CL. Spontaneous CL was more sensitive, but less reproducible. We purposely avoid the term background [33] and coin spontaneous CL. Induced CL compares to peak CL, except for some compounds possibly with predominant time-related effects.

The two PMNL-rich (24 hr PEC) and MØ-rich (96 hr PEC) murine phagocyte populations employed give largely similar results with the tested compounds in spite of the known difference in ROS release of PMNL and MØ [31, 34] and also of different responses of MØ from various sources [26, 33]. Species differences have also to be taken into account [35].

The phagocytes are activated by complement-opsonized zymosan (ZyC3b) which is thought to be of much greater biological significance than the often used chemotactic tripeptide FMLP [15, 36] or phorbol myristate acetate (PMA) [12]. Artificial enhancement of ROS generation using, for example, cytochalasin B, was purposely avoided [9, 16, 17]. The often cited results from Oyanagui [37, 38] employing lactate dehydrogenase-bound NADPH were not confirmed in this laboratory and by literature data [25, 27, 28, 39].

Arachidonic acid (AA) metabolism is one of the central phenomena of activated phagocytes and proceeds by enzymatic oxidation via the important cyclo- and lipoxygenase systems [14]. The interrelation of ROS generation and AA oxidation appears to be highly complicated, yet the mechanisms are still unresolved [40–44]. Prostaglandin synthetase probably releases ROS [7], and yet appears to be sensitive to oxidative self-inactivation [45]. This observation is corroborated by the finding that antioxidants may enhance PGE_2 and PGI_2 synthesis by protection of prostaglandin synthetase against oxidative self-inactivation [46].

The interrelation of AA metabolism and ROS generation is not only via direct oxygenation reactions, but most probably also via secondary phagocyte down- and up-regulation of the synthesis of cyclo- and lipoxygenase products. Whereas prostaglandins ($PGE_{1/2}$) inhibit ROS production, LTB_4 and 15-HPETE are stimulatory [26, 47]. Calcium-dependent processes are involved as well [25, 48]. That cyclooxygenase inhibitors (NSAID) do not effectively interfere with LgCL is confirmed by reports of a lack of inhibition of O_2^- generation [9, 49] and lack of antioxidant properties [50]. Compounds with lipoxygenase inhibitory properties belong to the most effective inhibitors of either LgCL and LuCL. O_2^- reduction by the lipoxygenase inhibitors eicosatetraynoic acid (ETYA) [49] nordihydroguaiaretic acid (NDGA) [51] and phenidone [14] and antioxidant effects are reported [50]. This implies a still undefined interrelation of both ROS generation and lipoxygenase activity [52, 53].

Cyclooxygenase inhibitors reduce the ROS generation only in H_2O_2-dependent systems such as LuCL and MPO-mediated LuCl [54], lactoperoxidase and horse radish peroxidase [55], and with xanthine oxidase-dependent effects on synovial fluid viscosity [56], but not with the xanthine oxidase itself [57*]. Yet, NSAID are ineffective with the nitroblue tetrazolium reduction test (NBT) [18]. These findings are in agreement with the preferential inhibition of LuCL, also reported by Bird and Giroud [27].

The most effective NSAID in LgCL is benzydamine (IC_{50} values between 10^{-5} and 10^{-4} mol/l). LuCL is less effectively inhibited, confirmed by others [39]. Benzydamine is neither a cyclo- nor a lipoxygenase inhibitor and is not cytotoxic at 10^{-4} mol/l [58*]. The known local anesthetic activity of benzydamine [59] and the inhibition of ROS generation may be related as for local anesthetic drugs [60, 61]. Carprofen has as yet not been reported to be a radical scavenger. Indomethacin was found to be inactive with LgCL using PMNL-rich 24 hr PEC, 96 hr PEC and also with LgCL for 96 hr PEC (not all data shown). With O_2^--dependent detection systems indomethacin was found to be ineffective [15, 18] (as we demonstrated with LgCL) or even stimulatory with LgCL [28]. H_2O_2-dependent LuCL was about 50% inhibited at 10^{-4} mol/l. This is an agreement with reports by Bird and Giroud [27] and is even more inhibited when rat resp. human PMNL are employed. LuCL stimulation has been reported with MØ and *ex vivo* with mouse PEC after 2 days pretreatment with 2 mg/kg/day p.o. indomethacin [62]. Similar results are also found with sulindac which has been described to be easily oxidizable by the prostaglandin synthetase and via MPO [63]. Piroxicam does not inhibit LgCL but slightly LuCL which is confirmed for LuCL [27]. O_2^--dependent inhibition of ferricytochrome *c* reduction *in vitro* has been reported with various stimulants of phagocytes [64] and has also been claimed for human PMNL *ex vivo* after 3 days pretreatment with 20 mg/kg/day p.o. [15]. Metamizol slightly enhances LgCL, but concentration-dependently inhibits LuCL. It inhibits neither lipoxygenase nor effectively cyclooxygenase, yet this NSAID is considered to be a prodrug. Thus, direct parallels cannot always be drawn between *in vitro* and *in vivo* systems [65]. Diclofenac enhances LgCL especially with unstimulated cells of both 24 hr (+24% at 10^{-4} mol/l) and 96 hr PEC (data not shown). Bird and Giroud [27] employing rat PMNL found a greater inhibition as we did with LuCL and murine PEC. Phenylbutazone is nearly ineffective in LgCL and slightly inhibitory in LuCL. The latter effect is possibly related to the reported MPO-mediated inhibition in LuCL [54]. Of interest is the specific antagonism of FMLP-stimulated ROS generation (11, 36, 39, 66]. Acetylsalicylic acid does not interfere with ROS-related CL confirmed by others also employing LuCL [25] and other techniques [15, 18, 49]. Ibuprofen is nearly inactive with both LgCL and LuCL also reported for nitroblue tetrazolium [67] and ferricytochrome C reduction [15]. Benoxaprofen differs from most NSAID by an activation of LuCL, confirmed with unstimulated human PMNL [68]. Thus, the disputed lipoxygenase inhibition [69] is not corroborated by CL inhibition.

Lipoxygenase inhibitors belong to the most inhibitory anti-rheumatics in respect to ROS generation of phagocytes. The dual cyclo- and lipoxygenase inhibitors BW 755C, enolicam, timegadine and CBS 1108/1114 are inhibitory in the amplified CL tests. The outstanding inhibition of both O_2^- (LgCL) and H_2O_2-dependent LuCL by NDGA is probably not only related to the lipoxygenase inhibitory property [70], but also to the direct inhibition of the O_2^--generating NADPH oxidase [51]. Yet, NDGA is inactive *in vivo* (unpublished results). The other laboratory tool phenidone preferentially inhibits LuCL compared to LgCL, confirmed for LuCL [26]. ROS inhibition and lipoxygenase inhibition are possibly responsible for the reported antipromotor activity [71], the antiproliferative [72] and topical anti-inflammatory effects [73]. BW 755C preferentially inhibits LuCL with both phagocyte populations (salt > base; unpublished) which has been confirmed by others [26, 54]. LgCL is less effectively inhibited. A mechanistic relationship to lipoxygenase inhibition appears attractive [74]. Inhibition of mitochondrial respiration [75] and calcium influx possibly contribute to the observed effects [76]. The interesting antiphlogistic effects *in vivo* can possibly be ascribed to the inhibition of AA metabolism and ROS generation by phagocytes. Enolicam differs from BW 755C in that it inhibits LgCL moderately and to a lesser degree LuCL. ROS reduction has provisionally been reported with canine PMNL [77]. The dual cyclo- and lipoxygenase inhibitor CBS 1108/1114 (two different salts) [70] inhibited only moderately LgCL and to a greater extent LuCL. A similar profile was observed for phenidone and BW 755C. The observed inhibition of ROS generation by timegadine has been confirmed in spite of a different technique and might be related to the lipoxygenase inhibition [78]. Ebselen, reportedly a glutathione protective compound [79] and a lipoxygenase inhibitor is found only slightly inhibitory with both amplifiers using unstimulated phagocytes (spontaneous CL) thus not confirming results reported by others [80].

Sulfasalazine demonstrates a concentration-dependent and remarkable similar inhibition with LgCL and LuCL comparable for all three test parameters. This is confirmed with the ferricytochrome *c* reduction test (5×10^{-4} mol/l: 62% inhibition) [81]. There is no interference with MPO-mediated iodination [8]. ROS and 5-lipoxygenase inhibition [82] may be operative in the treatment of inflammatory bowl disease and chronic polyarthritis.

Retinol is reportedly a weak lipoxygenase inhibitor [83, 84] yet, it does not inhibit ROS generation detected by LgCL or LuCL, furthermore an enhancement with human PMNL and LuCL has been reported [85]. Low level CL inhibition is consistent with $^1O_2$ quenching by β-carotene [86, 87].

* R. Müller-Peddinghaus, M. Wurl, G. Lenoir, A. Houben and J. Remacle, Einfluß von Benzydamin auf Bildung und Interaktion von reaktiven Sauerstoffspezies und die Zyklo- und Lipoxigenase. To be published.

Corticosteroids tested so far are virtually inactive also confirmed by others with LuCL [25] and other test systems [18, 54, 88].

Among the disease modifying drugs for the treatment of rheumatoid arthritis *chloroquine* exerted an interesting concentration-dependent inhibition of LgCL but to a lesser extent with LuCL. Although D-penicillamine was inactive with LgCL and LuCL, others have reported ROS inhibition with two other detection systems [89]. Monocytes from patients with rheumatoid arthritis are reported to generate an enhanced amount of ROS under treatment with disease modifying drugs [22].

Available antiarthrotic drugs are at present preferentially for parenteral application, e.g. SOD and GAGPS. The physiologic ROS protectant SOD appears effective to treat osteoarthritis [44]. Expectedly, SOD is more effective in LgCL than LuCL. For GAGPS is the finding of a time-dependent inhibition of LgCL and LuCL of great interest. The proteinase [90] and complement inhibitory properties of GAGPS are known [91]. Proteinase inhibitors might influence ROS generation by inhibiting a membrane-bound serine esterase [92].

The anti gout drug allopurinol appeared to be unable to interfere with O_2^- generation (LgCL), and this has been confirmed by others [93]. LuCL is also not inhibited by allopurinol. Colchicine is only effective for spontaneous CL, but ineffective with activated phagocytes, confirmed also for $^1O_2$-dependent low level CL [20] and corroborating the observation that ROS generation and phagocytosis follow the same stimulants but are mechanistically independent. Sulfinpyrazone is only slightly inhibitory with LgCL and LuCL, not very dissimilar to phenylbutazone. The reported beneficial effect of sulfinpyrazone on experimental infarction [94] might be related to ROS reduction by invaded PMNL.

DMSO up to 10^{-4} mol/l in LgCL and LuCL is inactive, i.e. is not a ROS scavenger in these test systems and may be used as a solvent. Results are so far confirmed as DMSO is considered to be an $OH^\cdot$ scavenger [95, 96] and $OH^\cdot$ is likely not to react with luminol or lucigenin [24].

Detection of the highly important, tissue damaging ROS by LgCL or LuCL and the pharmacological interference with the ROS generating mechanisms of phagocytes *in vitro* has led to new possibilities to characterize certain antirheumatic drugs as radical scavengers. Cyclooxygenase and more effectively lipoxygenase inhibiting NSAID interfere with H_2O_2-dependent LuCL. The O_2^--dependent LgCL is inhibited only by a few NSAID, by lipoxygenase inhibitors, chloroquine and GAGPS. Corticosteroids are found inactive with either test system. Thus amplified CL appears of considerable value to expand the possibilities of quantitative *in vitro* pharmacology as has been shown also for antimicrobial agents [97].

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REFERENCES

1. D. Roos, in *The Cell Biology of Inflammation* (Ed. G. Weissmann), p. 337. Elsevier/North-Holland Biomedical Press, Amsterdam (1980).
2. G. O. Till and P. A. Ward, *J. Free Rad. Biol. Med.* **1**, 163 (1985).
3. W. Troll, G. Witz, B. Goldstein, D. Stone and T. Sugimura, *Carcinogenesis* **7**, 593 (1982).
4. B. M. Babior, *J. clin. Invest.* **73**, 599 (1984).
5. N. Borregaard, J. H. Schwartz and A. I. Tauber, *J. clin. Invest.* **74**, 455 (1984).
6. R. P. Mason and C. F. Chignell, *Pharmac. Rev.* **33**, 189 (1982).
7. C. Deby, J. Pincemail, G. Deby-Dupont, P. Braquet and R. Goutier, in *Icosanoids and Cancer* (Eds. H. Thaler-Dao, A. Crastes de Paulet and R. Paoletti), p. 31, Raven Press, New York (1984).
8. R. J. Smith, B. J. Bowman and S. S. Iden, *Biochem. Pharmac.* **33**, 973 (1984).
9. J. E. Smolen and G. Weissmann, *Biochem. Pharmac.* **29**, 533 (1980).
10. Y. Bromberg and E. Pick, *Cell. Immunol.* **79**, 240 (1983).
11. A. Perianin, M. Torres, M.-T. Labro and J. Hakim, *Biochem. Pharmac.* **32**, 2819 (1983).
12. K. Taniguchi and K. Takanaka, *Biochem. Pharmac.* **33**, 3165 (1984).
13. R. Müller-Peddinghaus, D. Niepold and W. Hillebrand, *Int. J. Immunopharmac.* **7**, 567 (1985).
14. K. M. Mullane and R. I. Flower, *Mater Med. Pol.* **12**, 195 (1980).
15. H. B. Kaplan, H. S. Edelson, H. M. Korchak, W. P. Given, S. Abramson and G. Weissmann, *Biochem. Pharmac.* **33**, 371 (1984).
16. P. A. Ward, M. C. Sulavik and K. J. Johnson, *Am. J. Pathol.* **116**, 223 (1984).
17. C. Camisa, B. Eisenstat, A. Ragaz and G. Weissmann, *J. Am. Acad. Dermatol.* **6**, 620 (1982).
18. A. Wildfeuer, *Z. Rheumatol.* **42**, 16 (1983).
19. M. W. Whitehouse, in *Chemical Biology of Inflammation* (Eds. J. C. Houck and B. K. Forscher), p. 293. Pergamon Press, Oxford (1968).
20. Y. Niwa, T. Sakane and Y. Miyachi, *Biochem. Pharmac.* **33**, 2355 (1984).
21. R. C. Allen, in *The Reticuloendothelial System*, Vol. 2 (Eds. A. J. Sbarra and R. R. Strauss), p. 309. Plenum, New York (1980).
22. N. P. Hurst, B. Bessac and G. Nuki, *Ann. Rheum. Dis.* **43**, 28 (1984).
23. C. Dahlgren, H. Aniansson and K.-E. Magnusson, *Infect. Immun.* **47**, 326 (1985).
24. R. Müller-Peddinghaus, *Int. J. Immunopharmac.* **6**, 455 (1984).
25. K. van Dyke, C. van Dyke, J. Udeinya, C. Brister and M. Wilson, *Clin. Chem.* **25**, 1655 (1979).
26. M. J. Parnham, C. Bittner and J. Winkelmann, *Immunopharmac.* **5**, 277 (1983).
27. J. Bird and J.-P. Giroud, *J. Pharmac. Meth.* **14**, 305 (1985).
28. T. D. Horan, A. A. Noujaim and T. A. McPherson, *Immunopharmacol.* **6**, 97 (1983).
29. E. Pick, J. Charon and D. Mizel, *J. Reticuloendothel. Soc.* **30**, 581 (1981).
30. R. E. Schopf, J. Mattar, W. Meyenburg, O. Scheiner, K. P. Hammann and E.-M. Lemmel, *J. Immunol. Meth.* **67**, 109 (1984).
31. R. Müller-Peddinghaus, G. Hoppe and W. Schumacher, *Zbl. Vet. Med. B* **30**, 559 (1983).
32. H. Aniansson, O. Stendahl and C. Dahlgren, *Acta Path. Microbiol. Immunol. Scand. C* **92**, 357 (1984).
33. C. D'Onofrio and M.-L. Lohmann-Matthes, *Immunobiol.* **167**, 414 (1984).

34. S. Kitagawa, F. Takaku and S. Sakamoto, *J. Immunol.* **125**, 359 (1984).
35. C. P. Higgins, R. L. Baehner, J. McCallister and L. A. Boxer, *Proc. Soc. exp. Biol. Med.* **158**, 478 (1978).
36. R. D. Nelson, J. M. Gracyk, V. D. Fiegel, M. J. Herron and D. E. Chenoweth, *Blood* **58**, 752 (1981).
37. Y. Oyanagui, *Biochem. Pharmac.* **25**, 1473 (1976).
38. Y. Oyanagui, *Biochem. Pharmac.* **27**, 777 (1978).
39. M. Baggiolini, P. Mauderli, U. Näf and B. Catanese, *Agents Actions* **16**, 346 (1985).
40. R. V. Panganamala, H. M. Sharma, H. Sprecher, J. C. Geer and D. G. Cornwell, *Prostaglandins* **8**, 3 (1974).
41. J. A. Badwey, J. T. Curnutte and M. L. Karnovsky, *J. biol. Chem.* **256**, 12640 (1981).
42. F. A. Kuehl, Jr., E. A. Ham, R. W. Egan, H. W. Dougherty, R. J. Bonney and J. L. Humes, in *Pathology of Oxygen* (Ed. A. P. Autor), p. 175. Academic Press, New York (1982).
43. H.-J. Duchstein, *Archs. Pharm.* **318**, 127 (1985).
44. L. Flohé, H. Giertz and R. Beckmann, in *Handbook of Inflammation*, Vol. 5 (Eds. L. E. Glynn, J. C. Houck and G. Weissmann), p. 255. Elsevier, Amsterdam (1985).
45. R. W. Egan, J. Paxton and F. A. Kuehl Jr., *J. biol. Chem.* **251**, 7329 (1976).
46. J. R. Beetens, A.-E. van Hoydonck and A. G. Herman, *Archs. Int. Pharmacodyn.* **277**, 56 (1985).
47. R. L. Smith and M. J. Weidemann, *Biochem. biophys. Res. Commun.* **97**, 973 (1980).
48. R. C. Franson, D. Eisen, R. Jesse and C. Lanni, *Biochem. J.* **186**, 633 (1980).
49. G. M. Bokoch and P. W. Reed, *Biochem. biophys. Res. Commun.* **90**, 481 (1979).
50. Z. Duniec, J. Robak and R. Gryglewski, *Biochem. Pharmac.* **32**, 2283 (1983).
51. F. Rossi, V. Della Bianca and P. Bellavite, *FEBS Lett.* **127**, 183 (1981).
52. A. Jörg, P. A. Chavaillaz and H. J. Ziltener, *Klin. Wochenschr.* **62**, 494 (1984).
53. L. Levine, *Biochem. Pharmac.* **32**, 3023 (1983).
54. G. Pekoe, K. van Dyke, D. Peden, H. Mengoli and D. English, *Agents Actions* **12**, 371 (1982).
55. A. van Zyl and A. Louw, *Biochem. Pharmac.* **28**, 2753 (1979).
56. P. Puig-Parellada and J. M. Planas, *Biochem. Pharmac.* **27**, 535 (1978).
57. Y. Oyanagui, in *Pathology of Oxygen* (Ed. A. P. Autor), p. 99. Academic Press, New York (1982).
58. G. Segre and S. Hammarström, *Int. J. Tiss. Reac.* **7**, 187 (1985).
59. V. Cioli, C. Corradino and P. Scorza Barcellona, *Int. J. Tiss. Reac.* **7**, 205 (1985).
60. I. M. Goldstein, S. Lind, S. Hoffstein and G. Weissmann, *J. exp. Med.* **146**, 483 (1977).
61. S. L. Peck, R. B. Johnston Jr. and L. D. Horwitz, *J. Pharmac. exp. Ther.* **235**, 418 (1985).
62. M. J. Parnham and C. Bittner, in *Oxygen Radicals in Chemistry and Biology* (Eds. W. Bors, M. Saran and D. Tait), p. 859. De Gruyter, Berlin (1984).
63. H. W. Dougherty, A. Hen and F. A. Kuehl Jr., *Agents Actions Suppl.* **7**, 167 (1980).
64. H. S. Edelson, H. B. Kaplan, H. M. Korchak, J. E. Smolen and G. Weissmann, *Biochem. biophys. Res. Commun.* **104**, 247 (1983).
65. K. U. Weithmann and H.-G. Alpermann, *Drug Res.* **35**, 947 (1985).
66. C. Dahinden and J. Fehr, *J. clin. Invest.* **66**, 884 (1980).
67. E. G. Maderazo, S. P. Breaux and C. L. Woronick, *J. Pharmac. Sci.* **73**, 1403 (1984).
68. R. Anderson and G. Jooné, *Int. J. Immunopharmac.* **6**, 269 (1984).
69. J. A. Salmon, L. C. Tilling and S. Moncada, *Biochem. Pharmac.* **33**, 2928 (1984).
70. C. Bertez, M. Miquel, C. Coquelet, D. Sincholle and C. Bonne, *Biochem. Pharmac.* **33**, 1757 (1984).
71. S. M. Fischer, in *Icosanoids and Cancer* (Eds. H. Thaler-Dao, A. Crastes de Paulet and R. Paoletti), p. 79. Raven Press, New York (1984).
72. F. R. DeRubertis, P. A. Craven and R. Saito, *J. clin. Invest.* **74**, 1614 (1984).
73. J. M. Young, D. A. Spires, C. J. Bedord, B. Wagner, S. J. Ballaron, and L. M. de Young, *J. Invest. Dermatol.* **82**, 367 (1984).
74. G. A. Higgs, R. J. Flower and J. R. Vane, *Biochem. Pharmac.* **28**, 1959 (1978).
75. K. Kawai, J. Hasegawa, H. Shiojiri, Y. Nozawa, K. Tsurumi and H. Fujimura, *Experientia* **41**, 490 (1985).
76. F. C. Copp, G. A. Higgs, S. Moncada and K. G. Mugridge, *Br. J. Pharmac.* **80**, 611 (1983).
77. L. Liauw, T. Tjan, Y. Sakane, H. H. Oei, E. Ku and R. D. Robson, *Fedn Proc. Fedn Am. Socs. exp. Biol.* **43**, 1100 (1984).
78. F. Laghi Pasini, A. L. Pasqui, L. Ceccatelli and T. Di Perri, *Int. J. Tiss. Reac.* **6**, 9 (1984).
79. A. Müller, H. Gabriel and H. Sies, *Biochem. Pharmac.* **34**, 1185 (1985).
80. M. J. Parnham and S. Kindt, *Biochem. Pharmac.* **33**, 3247 (1984).
81. L. Molin and O. Stendahl, *Acta med. scand.* **206**, 451 (1979).
82. W. F. Stenson and W. Lobos, *J. clin. Invest.* **69**, 494 (1982).
83. J. C. Sircar and C. F. Schwender, *Prostagl. Leukotr. Med.* **11**, 373 (1983).
84. M. A. Bray, *Eur. J. Pharmac.* **98**, 61 (1984).
85. T. Kato, H. Wokalek, M. Ernst and E. Schöpf, *Archs Dermatol. Res.* **271**, 205 (1981).
86. T. W. Kensler and M. A. Trush, *Cancer Res.* **41**, 216 (1981).
87. G. W. Burton and K. U. Ingold, *Science* **224**, 569 (1984).
88. L. K. Lim, N. H. Hunt and M. J. Weidemann, *Mol. Immunol.* **22**, 1365 (1985).
89. I. A. Rajkovic and R. Williams, *Biochem. Pharmac.* **33**, 1249 (1984).
90. M. Stancíková, K. Trnavsky and H. Keilová, *Biochem. Pharmac.* **26**, 2121 (1977).
91. H. P. Heinz and M. Loos, Schweizerisch-Deutscher Rheumatologenkongreß 5–9 Oktober 1982, Basel, p. 155.
92. M. Kudoh, T. Nakamura and J. Koyama, *Mol. Immunol.* **22**, 1099 (1985).
93. H. P. Jones, M. B. Grisham, S. K. Bose, V. A. Shannon, A. Schott and J. M. McCord, *Biochem. Pharmac.* **34**, 3673 (1985).
94. M. Karmazyn, *Br. J. Pharmac.* **83**, 221 (1984).
95. R. V. Panganamala, H. M. Sharma, R. E. Heikkila, J. C. Geer and D. G. Cornwell, *Prostaglandins* **11**, 599 (1976).
96. J. E. Repine, J. W. Eaton, M. W. Anders, J. R. Hoidal and R. B. Fox, *J. clin. Invest.* **64**, 1642 (1979).
97. R. A. Fromtling and G. K. Abruzzo, *Meth. Find. Exptl. Clin. Pharmac.* **7**, 493 (1985).